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ERRATUM.

P. 173, l. 34, "lung" should read "blood."

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TRANSMUTATIONS WITHIN THE STREPTOCOCCUS-PNEUMOCOCCUS GROUP.*

E. C. ROSENOW.

(From the Memorial Institute for Infectious Diseases, Chicago.)

(WITH PLATE 1.)

Changes in fermentative, morphological, and other properties of certain streptococci, particularly the hemolytic streptococcus, have been noted by Ruediger,[1] Anthony,[2] Walker,[3] and especially by Davis[4] and by Buerger and Rittenberg.[5]

Davis concludes that the "transformation of one member into another within certain limits appears to be not an uncommon phenomenon." Buerger and Rittenberg converted atypical pneumococci ("streptococcus cultural type") into typical pneumococci by animal passage. They were unable, however, to convert typical hemolytic streptococci into pneumococci and vice versa. Davis and Rosenow have shown that the encapsulated streptococcus from "septic sore throat" can be converted into *Str. mucosus* on the one hand and hemolytic streptococcus on the other. Hemolytic streptococci when soaked in unheated cows' milk, obtained in a

* Received for publication September 19, 1913.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 663.

[2] *Ibid.*, 1909, 6, p. 332.

[3] *Proc. Royal Soc.*, 1911, S.B. 83, p. 541.

[4] *Jour. Infect. Dis.*, 1913, 12, p. 386.

[5] *Ibid.*, 1907, 4, p. 609.

sterile manner,[1] take on cultural and other features resembling streptococci obtained from cases during a milk epidemic of sore throat, and when later passed through animals they take on the features of *Str. mucosus*.

Beattie and Yates[2] were unable to obtain permanent, specific fermentative reactions for streptococci. Levy[3] was unable to separate certain strains of *Str. viridans* from hemolytic streptococcus and considers *Str. mucosus* a variety of pneumococcus. Heinemann[4] has shown that "*Str. lacticus*," by animal passage, may take on features similar to hemolytic streptococci. Koch and Pakschischewsky[5] have shown that certain equine streptococci (Druse-Streptococcus), while not identical in all respects, resemble very closely indeed human virulent *Str. longus*.

In previous papers[6] I have shown that *Str. viridans* isolated chiefly from the blood in cases of subacute endocarditis and obtained also from the throat and other sources may by animal passage take on the properties of typical pneumococci; and hence I designated them as modified pneumococci. During a study on autolysis of pneumococci in salt solution and the effect of sodium oleate and bile on virulent pneumococci their transformation into hemolyzing streptococci was observed.

The anaphylactic reaction indicates that the chemical compositions of the various members of the group are similar. I have shown[7] that extracts of hemolytic streptococcus sensitize guinea-pigs to extracts of the pneumococcus and *Str. mucosus*, and vice versa. Davis,[8] working with heated suspensions of the various organisms, recently had similar results and concludes that the anaphylactic reaction is of little value in differentiating the various members of the streptococcus group.

It should be pointed out here, however, that pneumococci and hemolytic streptococci, for example, call forth other immunity reactions (opsonins and agglutinins) which are specific and which serve to separate them into distinct classes or types.

[1] Rosenow, *Jour. Infect. Dis.*, 1912, 11, p. 338.
[2] *Jour. Path. and Bact.*, 1911, 16, p. 246.
[3] *Virch. Arch.*, 1907, 157, p. 327.
[4] *Jour. Infect. Dis.*, 1907, 4, p. 89.
[5] *Ztschr. f. Hyg. u. Infectionskrankh.*, 1913, 74, p. 1.
[6] *Jour. Infect. Dis.*, 1909, 6, p. 245; *ibid.*, 1910, 7, p. 411.
[7] *Ibid.*, 1911, 9, p. 190.
[8] *Ibid.*, 1913, 12, p. 386.

Modifications in the human subject have also been observed. Buerger and Rittenberg found that the pneumococci obtained from the blood in puerperal sepsis are quite different from those obtained from metastatic abscesses in the same case. I have made similar observations in acute otitis and in pneumonia[1] that suggest the transformation *in vivo* of *Str. mucosus* and of pneumococcus into hemolytic streptococcus. Toenniessen[2] has produced mutational forms in case of *B. mucosus*. It does not appear, however, that it has been shown that typical hemolytic streptococci from scarlet fever, for example, can be converted into typical pneumococci. The "pure line" requirement—that is, working with a strain obtained from single individuals—of students of heredity has not been met except in the case of the transformation of *Str. viridans* into pneumococcus as mentioned.

A more elaborate study of this question, therefore, seemed desirable.

In the experiments now to be described, growth on the surface of human blood agar was used as a criterion of change. The standard agar was prepared from Liebig's extract of beef and Witte's peptone; approximately 0.5 c.c. of sterile defibrinated human blood was added to each tube of 7 c.c. of melted and cooled agar just before pouring the plates.

EXPERIMENTS WITH VARIOUS STREPTOCOCCAL STRAINS.

July 28, 1911. Strain 595 was isolated from the tonsil in acute tonsillitis of scarlet fever. A practically pure culture of hemolyzing streptococci was obtained on blood agar plates. One typical hemolyzing colony was selected and plated again and a single colony from the second plating used for the subsequent work. From this date until June, 1912, it was grown on human blood agar. During this time it was plated out three times, and it always yielded only hemolyzing colonies, and the growth on blood agar slants always caused hemolysis. It produced arthritis in rabbits.

June 29, 1912. Subculture was made on blood agar and the tube sealed with a cork previously soaked in paraffin.

October 8. Culture on blood agar plates, hemolyzing colonies only. The amount of hemolysis was approximately one-third as great as when first isolated. Inoculations were made from single colonies on two blood agar slants containing peptone and extract of beef. These uncorked tubes were kept at 37° C. until November 6. On this date the media were very dry. Inoculations on the usual blood agar slants were made and placed at 37° C.

[1] *Jour Infect. Dis.*, 1911, 8, p. 500

[2] *Centralbl. f. Bakteriol.* I, *Orig.* 1913, 6, p. 391

November 15. Subcultures on the surface of blood agar plate the day before yielded two kinds of colonies, one larger, producing the usual zone of hemolysis, the other smaller and producing no hemolysis. Subcultures were made on the surface of blood agar plates from the two types of colonies.

November 17. The larger colonies yielded only hemolyzing colonies while the smaller yielded only small, green, tightly adherent colonies with no trace of hemolysis. Subcultures of each were made on the surface of Löffler's serum and four colonies of each variety plated on blood agar plates.

November 19. The subcultures of each of the four hemolyzing colonies yielded only hemolyzing colonies. Three of the adherent colonies yielded only adherent green colonies, while the other yielded adherent and non-adherent green colonies in about equal proportions, but no hemolyzing colonies. Duplicate cultures on blood agar slants from a single hemolyzing and a single adherent green colony.

November 21. Hemolyzing colony yielded hemolyzing growth, the green colony a non-hemolyzing, adherent, green growth. Subcultures of each were made on Löffler's serum and blood agar slants, sealed and placed at 37° C.

The hemolyzing strain produced a diffuse turbidity in ascites broth together with some sediment, the green-producing strain, no turbidity but an abundant sediment. Smears of the hemolyzer showed diplococci and short chains, of the green-producer, both large and small diplococci and very long chains and clumps. Any diplococcus arrangement in the chains of the hemolyzer was hard to make out (Fig. 1); in the green-producer the diplococci were very distinct, elongated, and often lanceolate in shape (see Fig. 2). Neither strain fermented inulin. The hemolyzer fermented mannite, the green-producer did not; the former failed to ferment maltose and saccharose, the latter did; both precipitated serum-dextrose agar, the hemolyzer more markedly; neither produced acid in pneumonic serum; neither was soluble in rabbit bile.

If the modified strain which adhered to the surface and produced the green zone around the colonies was really *Str. viridans*, as seemed likely, then it should have produced characteristic lesions in animals. Accordingly, on November 19, the growth from 20 to 60 c.c. of ascites-dextrose broth suspended in NaCl solution was injected in the ear vein of four small rabbits (600 to 750 gms.). Two other rabbits were injected with comparable doses of the corresponding hemolyzing strain. Previously I have shown that the ability of *Str. viridans* to produce valvular hemorrhages and endocarditis depends definitely on the property to form long chains and clumps. Hence two of the four rabbits were injected with a thoroughly shaken suspension in which only small clumps of organisms could be found in smears and two with the clumped suspension the result of prolonged centrifugation. All the rabbits died within 36 hours. The two injected with the shaken suspension showed small tricuspid hemorrhages but no hemorrhages anywhere else. The two injected with the clumped suspension showed large tricuspid and papillary hemorrhages, and hemorrhages in the glomerular tufts, and one showed hemorrhages in the mucous membrane of the pyloric end of the stomach. The two injected with the hemolyzing strain did not show valvular hemorrhages but subendocardial hemorrhages of the septum in the left ventricle. Cultures from the blood of the four injected with the green-producer showed a few green colonies in three; the joints in two showed a few green colonies while in the others they were sterile. The two injected with the hemolyzing variety showed a large number of hemolyzing colonies from the joints and a moderate number from the blood. Subcultures of each were made on blood agar slants.

The effect of clumping and the a... ...
the green-producing variety and for joints of ... ...ing variety are well illustrated by this experiment. In this case the transformation of a hemolyzing streptococcus to a green-producing streptococcus occurred on blood agar with peptone and beef extract, the supply of oxygen being abundant.

There now follows an illustration of another method by which transformation may be brought about.

January 29, 1913. A blood agar plate which was inoculated with the growth of hemolytic streptococcus (No. 683) became contaminated accidentally with three colonies of what was apparently *B. subtilis*. The plate contained mostly hemolyzing colonies but there was found in addition a number of green, moderately adherent colonies. These were all in relatively close proximity to the colonies of the bacilli. Smears of the green colony showed small gram-staining diplococci, often in clumps and chains. The large colonies were large, gram-staining bacilli.

Subcultures were now made on blood agar plates of two hemolyzing and two green colonies and from two of the bacillary colonies.

January 31. The green colonies produced green colonies, the hemolyzing colonies, hemolyzing colonies, and the *B. subtilis* plate showed no green nor hemolyzing colonies.

February 5. Subcultures of the hemolyzing and green-producing colonies of Strain 595 were made on the surface of blood agar plates, one of which was inoculated also with a dilute culture of *B. subtilis*.

February 6. The plates containing the hemolyzing strain showed only hemolyzing colonies, while the set of this strain inoculated also with *B. subtilis* showed both hemolyzing and green, adherent colonies; the latter exclusively in a zone within a radius of approximately 0.5 cm. around the *B. subtilis* colonies. Approximately one-half of the colonies inside of this zone were green, adherent, and non-hemolyzing.

Subcultures of the non-hemolyzing strain which was made on November 21, 1912, on blood agar still yielded only green colonies while those from Löffler's serum yielded three-fourths green and one-fourth hemolyzing colonies.

In order to make it even more certain that the green colonies on the *B. subtilis* plate were not accidental, four hemolyzing colonies were plated on the surface of blood agar in such a way that one-half of the plate contained only streptococcus colonies while the other half contained *B. subtilis* as well. In the same way subcultures were also made from blood agar slants and this time with a strain of *B. subtilis* which had been plated out from single colonies four times and had always yielded only *B. subtilis*. The hemolyzing colony and the green colony on the surface of blood agar slants bred true. Each hemolyzing colony gave only hemolyzing colonies on the half of the plate containing no *B. subtilis* while approximately two-thirds of the colonies in the other half were adherent, green colonies. The part of the plates made from the blood agar slants without *B. subtilis* yielded only hemolyzing colonies; the parts inoculated with both yielded both varieties of colonies. The green colonies again appeared in rather close proximity to *B. subtilis* colonies.

The following experiments illustrate the affinity of the two varieties of streptococci after one animal passage for the valves of the heart and the joints respectively.

.. The organisms, grown in ascites-dextrose broth, [illegible] poured off, and the organisms suspended in NaCl solution so that 1 c.c. represented 10 c.c. broth culture.

Rabbit 221 (535 gms.).—

November 26. Injected with the growth from 25 c.c. of broth of the green-producing strain after one animal passage.

November 27. Seemed quite well.

November 28. Killed; no gross lesions except two hemorrhages in tricuspid valve. Smears from blood and joints negative. Blood agar plate cultures from blood, knee joints, and from surface of valve all sterile. From the tissue of the valve about 150 green colonies developed.

Rabbit 322 (630 gms.).—

November 26. Injected with the growth from 40 c.c. of broth of the green-producing strain after one animal passage. Some dyspnea soon after injection.

November 27. Seemed quite well.

December 1. Seemed ill.

December 3. Loss of weight, had grown blind from an opacity and turbidity of anterior chamber due to conjunctivitis. Chloroformed. Large vegetative tricuspid endocarditis; multiple, white miliary nodules in cortex of kidney. Smears from vegetations, from renal lesions, and anterior chamber of eye show many gram-staining diplococci and clumps. Blood and joint fluids sterile. Large number of green-producing colonies developed from vegetation, from kidneys, and from the anterior chamber.

Rabbit 323 (575 gms.).—

November 26. Injected with growth from 40 c.c. of broth of the hemolyzing strain after one animal passage. Slight dyspnea soon after injection.

November 27. Dead. No gross lesions but some fluid in peritoneal cavity containing hemolyzed blood. Large number of hemolyzing colonies from blood and moderate number from joints and peritoneal fluid.

Since it has been shown that the usual viridans-strains do not produce suppuration when injected subcutaneously and do not cause diffuse peritonitis when injected intraperitoneally, it was thought worth while to test the properties of these two strains in this respect. Four guinea-pigs were injected with comparable doses of the green-producing and hemolyzing varieties before animal passage. The green-producer disappeared rapidly and the animals recovered, while the hemolyzer increased in the blood and caused death with general peritonitis.

From these experiments it would seem, then, that the green-producer really was *Str. viridans*. If this was true, then animal passage should have converted it into a pneumococcus just as I have shown to be the case with *Str. viridans* obtained from human sources. A strain from a single colony from one of the rabbits injected November 19 was selected for this purpose. In order to guard against the loss of the organism in the animals and the possibility of obtaining pneumococci from sources in the animal other than the organisms injected, the first passages were

carried out in duplicate, the animals from absolutely healthy stock were caged separately, and blood agar plate cultures were made from the blood and peritoneal exudate at intervals during life and as soon after death as possible.

Guinea-pig 993.—

November 23. Injected intraperitoneally with the growth from 40 c.c. of ascites-dextrose broth of the strain from Rabbit 595-G^2.

November 24. Dead; mild serofibrinous peritonitis. Smears from blood showed no organisms while those from peritoneal exudate showed many leukocytes, diplococci, and chains. There was marked phagocytosis. Cultures from the blood and peritoneal exudate; 1.5 c.c. of the latter were injected intraperitoneally in Guinea-pig 996.

November 25. Cultures from blood showed a few while those from the peritoneal exudate showed many green colonies in pure growth. Guinea-pig 996 dead; serofibrinous peritonitis and pleuritis. Peritoneal smears showed more marked phagocytosis than those from the pleural cavity. Cultures made and 1 c.c. of peritoneal exudate injected intraperitoneally in Guinea-pig 997.

November 26. Cultures from blood, peritoneal and pleural fluids, gave a pure culture of green-producers, the colonies being distinctly larger and more moist than before inoculation, but without capsules.

November 27. Guinea-pig 997 dead; serofibrinous peritonitis. Smears from the exudate showed less phagocytosis, few chains, fewer leukocytes; a few diplococci found in the blood. Pure culture of green colonies from blood and peritoneal fluid. Subcultures from the blood on surface of blood agar slant and in ascites-dextrose broth gave growths that on November 29 appeared green on transmitted light and quite moist; capsule stain negative. In the broth diffuse turbidity with some sediment, consisting entirely of non-encapsulated diplococci and short chains. Fresh subcultures on the surface of blood agar slants, in inulin broth and on inulin agar, on ascites-dextrose agar and in pneumonic serum.

Guinea-pig 1003.—

November 30. Injected intraperitoneally with the surface growth of one blood agar slant.

December 1. Seemed ill.

December 2. Dead; serofibrinous peritonitis and pleuritis. Smears gave diplococci in rather large numbers in the blood, some of which were distinctly encapsulated, and very many in the peritoneal and pleural exudate. Phagocytosis now much less than in the previous animals. Cultures from the blood on the surface of blood agar slant and plate and on the latter from the peritoneal and pleural exudate.

The strain from Guinea-pig 997 (595-G^4) ferments inulin broth, no longer precipitates ascites-dextrose agar, but does not produce acid in pneumonic serum as do typical pneumococci.

Guinea-pig 1007.—

December 3. Injected intraperitoneally with the surface growth of one blood agar slant obtained from the blood of Guinea-pig 1003.

December 4. Dead; serofibrinous peritonitis, pericarditis, and pleuritis. Smears gave encapsulated diplococci in exudate and blood. Very little phagocytosis. Subcultures in the usual way.

Similar experiments were repeated until the strain had been passed through 14 animals. It had now become so virulent that 0.1 c.c. of a broth culture killed in 24 hours by producing bacteremia. At this time the organisms were found encapsulated in the blood, in the exudate as well as on blood agar and in ascites broth cultures. Morphologically they were indistinguishable from typical pneumococci (see Fig. 3). The strain now fermented inulin broth and agar, did not cloud ascites-dextrose agar, produced acid in pneumonic serum (see Table 1), was soluble in bile, and autolyzed, as pneumococci do, in NaCl solution under ether. Its broth-culture filtrate behaved like pneumococcus broth-culture filtrates and while the strain failed to grow in pneumococcus broth-culture filtrates it grew readily in streptococcus broth-culture filtrates (Marmorek's test). Table 1 shows the fermentative and other cultural characteristics of Strain 595 as a streptococcus and as a pneumococcus on March 11, 1913. Similar results have been obtained at other times.

TABLE 1.

The Fermentative and Other Features of Strain 595 as a Streptococcus and as a Pneumococcus.*

Media	Strain 595					
	As Streptococcus			As Pneumococcus		
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
Blood agar	H	H	H	G	G	G
Serum dextrose agar	+	+	++	o	o	o
Inulin broth	o	o	o	+	+	++
Inulin agar	o	o	o	o	+	+
Pneumonic serum	o	o	o	+	+	+
Mannite	o	o	o	o	o	o
Saccharose	o	o	+	+	+	+
Dextrose	+	+	+	+	+	+
Lactose	o	o	+	o	o	o
Maltose	o	o	o	o	o	+

* In the various tables, H stands for hemolysis and G for green. + stands for clouding of serum dextrose agar, acid production in pneumonic serum, and for fermentation of the various sugars. o stands for no clouding of serum dextrose agar, no acid production in pneumonic serum, and no fermentation of the various sugars.

The original hemolyzing strain has been grown on blood agar continuously and has remained unaltered. The strain as a pneumococcus has also been continuously grown on blood agar slants with cotton plugs. It has remained virulent and as a pneumococcus in the cultures; transfers have been made once or twice a week

for six months. However, a subculture made five months after the transformation had taken place from an old blood agar slant which had become quite dry from evaporation, yielded a hemolytic, relatively avirulent streptococcus similar to the original growth. The fermentative and other cultural properties of this strain after reversion from pneumococcus were the same as before its transformation into a pneumococcus except that it now fermented mannite and maltose. It had lost its capsule and again become susceptible to phagocytosis by human leukocytes in human serum. Its ability to grow in the blood stream and to kill by streptococcemia had disappeared while the affinity for joints had returned.

Two other important alterations have been observed in this strain. The strain as a pneumococcus after 14 animal passages, and two other strains of pneumococci and two of streptococci were inoculated February 7 into two sets of tubes containing dialyzed beef serum agar slants. To these there were added varying amounts of Ringer's solution. One set was placed at 37° C., the other kept at room temperature. Subcultures on the surface of blood agar plates were made at the end of 10 days. No noteworthy changes in the character of growth on blood agar were noted in the tubes made isotonic with two and five times the strength of normal Ringer's solution but in the tubes containing 10 times the normal of Ringer's solution at 37° C. this and one of the other strains of pneumococcus, namely No. 683, had taken on the characteristics of *Str. viridans*, and in the tubes containing 20 times the strength of normal Ringer's solution, at room temperature, one streptococcus had become altered so that subcultures yielded very small pin-point colonies surrounded by a wide zone of hemolysis, the smears showing very small gram-staining diplococci and chains exactly similar to certain strains of streptococci obtained not infrequently from the crypts of extirpated tonsils. Cultures were made from three colonies on the surface of blood agar plates. Each yielded only the small colonies and cocci.

If Strain 595 was really a streptococcus and the one formed from it really a pneumococcus, then the former should have produced specific antibodies for streptococci and the latter specific antibodies for pneumococci.

In order to test this point two rabbits, Nos. 458 and 459, weighing 750 gms. each, were injected intraperitoneally, April 3, with the heated (60° C. 30 min.) suspensions in NaCl solution from two blood agar slants, Rabbit 458 with the streptococcal strain, and Rabbit 459 with the pneumococcal strain. Samples of blood from each were taken just before injection, for three consecutive days, and on the fifth day following the injection. The sera were kept frozen in a vacuum bottle until April 9 when the opsonic index was determined as indicated in Charts 1 and 2. The counts were made by individuals without knowledge of the dates when the sera were obtained or the

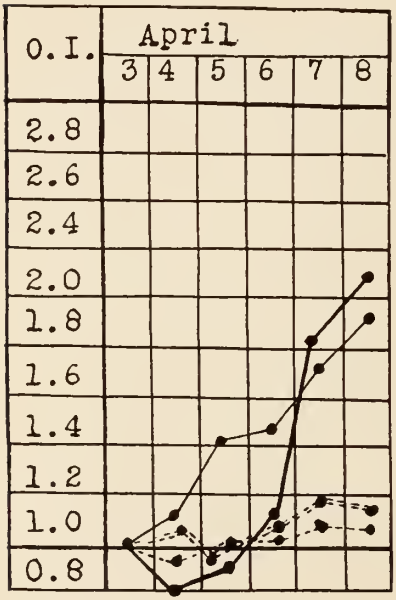

CHART 1.—Opsonic index of serum of rabbit immunized with Strain 595 as a streptococcus.

Homologous streptococcus.
Heterologous streptococcus.
Homologous strain as pneumococcus.
Heterologous pneumococcus.

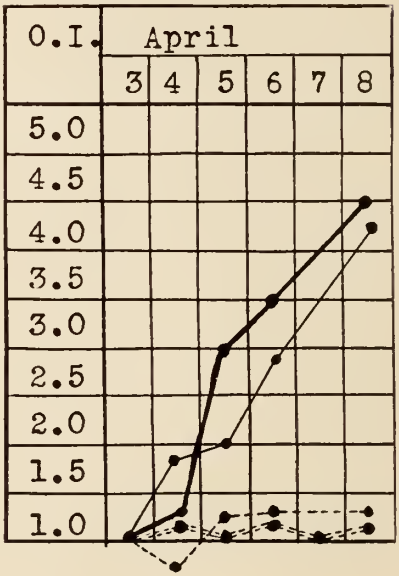

CHART 2.—Opsonic index of serum of rabbit immunized with Strain 595 as a pneumococcus.

Homologous pneumococcus.
Heterologous pneumococcus.
Homologous strain as streptococcus.
Heterologous streptococcus.

strains used. The charts show that the strain as a streptococcus caused a specific increase in opsonin for homologous and one other strain of hemolytic streptococcus, but no perceptible increase for the homologous strain as a pneumococcus nor for another strain of pneumococcus. The strain as a pneumococcus on the other hand caused a specific rise in opsonin for both the homologous and another strain of pneumococcus but not for the homologous strain as a streptococcus nor for another streptococcus. In order to check these results the rabbits were injected with comparable doses (growth from two blood agar slants) on April 15, 17, and 18, and were then bled on April 26. The point of opsonic extinction of the serum of each and a normal rabbit as control was now determined. The serum from the rabbit immunized with Strain

595 as a streptococcus gave opsonic extinction for the homologous and another strain of hemolytic streptococcus at a dilution of 1–192; for Strain 595 as a pneumococcus and for another pneumococcus at 1–12. The serum from the rabbit immunized with Strain 595 as a pneumococcus showed opsonic extinction at a dilution of 1–192 for the homologous and the other strain of streptococcus at 1–48. The serum from the normal rabbit gave opsonic extinction for the streptococci at 1–48 and for the pneumococci at 1–12. Counts were made also of the cocci taken up in the various dilutions, 50 leukocytes being counted. The results, given in Table 2, the opsonic index, and the point of opsonic extinction show that the serum from the rabbit immunized with Strain 595 as a hemolytic streptococcus contained an increase in specific opsonin for streptococci and the serum from the rabbit immunized with Strain 595 as a pneumococcus an increase in specific opsonin for pneumococci.

TABLE 2.

THE OPSONIC POWER OF THE SERUM OF NORMAL AND IMMUNIZED RABBITS.

SERUM	NUMBER OF ORGANISMS TAKEN UP IN THE VARIOUS DILUTIONS IN DETERMINING THE POINT OF OPSONIC EXTINCTION			
	Streptococcus 595	Streptococcus M	Pneumococcus 595	Pneumococcus 678[19]
Rabbit immunized with Strain 595 as a streptococcus	112	159	8	6
Rabbit immunized with Strain 595 as a pneumococcus	65	82	45	26
Normal rabbit control	59	76	6	7

Strain XII was isolated as a typical hemolytic streptococcus from a single colony on a blood agar plate (June 2, 1912) inoculated with the blood of a guinea-pig which died from a streptococcemia 48 hours after the injection of a suspension of separator slime. The growth from one blood agar slant was injected intraperitoneally into a rabbit which died in 24 hours with peritonitis. The blood agar plate inoculated with the blood showed a pure culture of markedly hemolyzing colonies. A subculture from a single colony was made and the strain cultivated continually on blood agar slants which were closed with paraffin corks until January 19, 1913. From blood agar plates now made only hemolyzing colonies were obtained. A single colony was used in inoculating two blood agar slants. One strain has been cultivated on blood agar in the usual way continuously since. It has been plated out on blood agar repeatedly and has always produced only hemolyzing colonies. The amount of hemolysis, however, is approximately one-half as great as it was at first. The other strain was used for this work. By growing it for three generations on ascites-dextrose agar in pure oxygen and then plating on blood agar plates three types of colonies were obtained: approximately one-half of the colonies produced a slight and hazy hemolysis; one-third were grayish, non-adherent colonies which did not affect the media; and then there were some green, tightly adherent colonies. The slightly hemolyzing colonies showed diplococci and short chains, the gray colonies small micrococci and an occasional short chain. These growths closely corresponded, culturally and morphologically, to two types of the organisms obtained from rheumatism. The adherent green colonies showed long chains and clumps of small elongated diplococci similar to those shown in Fig. 2. Plate cultures from three of each of these varieties showed that each bred true and a single colony of the green variety was selected for animal passage.

The growths from 20 to 60 c.c. of ascites-dextrose broth were injected intravenously successively in five rabbits. Valvular hemorrhages were found in two after two and three passages respectively, and vegetative endocarditis in two, in the fourth and fifth passage. The organism was now injected intraperitoneally in guinea-pigs, and cultures were made on blood agar plates after death or after 48 hours if recovery seemed likely. After three passages in this way the organism had become quite virulent, producing peritonitis, and had most of the characteristics of a pneumococcus but had no demonstrable capsule and still formed chains. It now fermented inulin, produced acid in pneumonic serum, no longer clouded ascites-dextrose broth, autolyzed in NaCl solution, and dissolved in bile. After 12 animal passages its virulence was so great that 0.1 c.c. of ascites-dextrose broth culture injected intraperitoneally killed a guinea-pig in 16 hours. The blood and cultures now showed organisms with a wide, stainable capsule that corresponded to all the cultural and agglutination tests of pneumococci. After 14 animal passages the capsule had become still more marked, the growth on blood agar and ascites-dextrose agar was now mucoid in character, and the organism corresponded to *Str. mucosus* in every respect. At first the guinea-pigs had a rather mild peritonitis, later a marked serofibrinous peritonitis and pleuritis and still later also pericarditis, when as a *Str. mucosus* the exudates were mucoid in character. After five rabbit (intravenous injection) and two guinea-pig (intraperitoneal injection) passages it produced death in a rabbit in 22 days from a general anasarca due to an obstructive vegetative endocarditis of the tricuspid valve, cultures from the vegetation yielding an organism resembling in every way *Str. viridans*. After nine animal passages its affinity for the endocardium had disappeared and it now produced pulmonary hemorrhages and acute peribronchial lymphadenitis on intravenous injection in rabbits. This strain has been cultivated continuously on blood agar for six months, making transfers once or twice a week. It has lost its mucoid character but has retained its capsule, virulence, and the other characteristics of a pneumococcus.

The hemolyzing variety of this strain was converted into *Str. viridans* also by growth in oxygen, by growth in symbiosis with *B. subtilis*, in salt free broth, and in hypertonic broth.

Strain 734 was isolated January 27, 1913, from the joint of a case of acute articular rheumatism. This organism as isolated produced long chains in broth (Fig. 4), green on blood agar, did not ferment mannite nor inulin, and when injected intravenously in rabbits produced multiple arthritis, endocarditis, and pericarditis. In an attempt to modify this strain it was inoculated (February 13) into sterile urine, distilled water, and on blood agar slants. One set of the tubes was kept under aerobic, the other set under anaerobic, conditions at 37° C., and a blood agar slant in oxygen as well. Subcultures on blood agar plates were made March 1. The tube of blood agar kept in oxygen and the anaerobic urine tube were sterile. The others all yielded green colonies but the amount of green was distinctly less and the organisms small in the cultures from the urine. The aerobic culture in distilled water yielded 11 small hemolyzing colonies. When this strain was now grown in broth it produced a diffuse turbidity, diplococci, and short chains. It had acquired the power to ferment mannite and when injected intravenously in rabbits produced now a myositis and myocarditis in addition to arthritis and endocarditis and often focal nephritis. Its virulence was distinctly greater than before. This was manifested by the greater range of lesions produced, by its slower disappearance from the circulation, and by the fact that it

killed rabbits in smaller doses. From the pericardium of a rabbit which was injected with this strain after two animal passages, there was isolated a number of green-producing colonies. This green-producing strain was now injected intraperitoneally into guinea-pigs. Its virulence increased rapidly and after five animal passages it had acquired all the features of a moderately virulent pneumococcus. After 10 animal passages this strain had become exactly like *Str. mucosus*. The strain as isolated was also transformed into a typical pneumococcus but it required 11 animal passages. Both strains lost completely their original affinity for joints, muscles, etc., and at a certain grade of virulence produced, on intravenous injection, pulmonary hemorrhages and bronchopneumonia while still later they caused death from pneumococcemia irrespective of the place of injection.

The strain which produced green and which has been kept on blood agar now produces small dry greenish colonies. Its affinity for joints has disappeared, while its power to produce vegetative endocarditis has increased. It is quite like *Str. viridans* in every respect.

Strain 736 was isolated March 20, 1913, from the joint in a case of acute articular and muscular rheumatism. This strain resembled in every way the strain just mentioned after it had acquired slight hemolyzing powers (see Fig. 5). It produced a narrow zone of a hazy hemolysis and when injected into the ear vein of rabbits it produced myositis, myocarditis, arthritis, and endocarditis. In the joint of one rabbit in its second passage it lost the power to hemolyze and now produced green colonies instead. It did not yet ferment inulin nor produce acid in pneumonic serum. This strain had lost most of its affinity for the muscles, endocardium, joints, and kidney, and now produced acute pericarditis, acute splenitis, and a bronchopneumonia. After six passages in guinea-pigs it had all the characteristics of a pneumococcus. The hemolyzing strain during the second passage produced a moderate number of muscle lesions as well as myocarditis and endocarditis, a few in the third, but after the fourth and fifth passages the muscles, myocardium, endocardium, and kidney were no longer involved. It produced ulcer of the stomach in the second and third passages but, after subsequent passages, a suppurating arthritis instead. The strain which has been cultivated continuously on blood agar has acquired greater hemolyzing power. One month after isolation plate cultures from four of the colonies near the upper end of one of the tubes of ascites-dextrose agar, which was inoculated with joint fluid, show these organisms to be changed whereas each of the colonies in the depths yields two kinds of colonies: one similar to the colonies above and the other non-hemolyzing, more opaque, grayish colonies. Smears from these show small micrococci in clumps and an occasional short chain. This strain now resembles the third type from rheumatism. Similar results were obtained with two other strains from rheumatism.

Strain B was isolated April 18, 1912, as *Str. viridans* from the blood during life in a case of subacute infectious endocarditis. A "pure line" was obtained for further study. It produced valvular hemorrhages in five and endocarditis in two rabbits soon after isolation. After cultivation on blood agar for nearly a year it acquired the power to hemolyze blood agar, lost its affinity for the endocardium, but acquired an affinity for joints. Cultures from the joint, in one rabbit, showed both green and hemolyzing colonies. Cultures from the former were injected successively into four guinea-pigs. It now acquired a distinct capsule and the power to ferment inulin, to produce acid in pneumonic serum, and to dissolve in bile.

Strain 319 was isolated December 11, 1907, from the blood during life in a case of lobar pneumonia. It was passed through one guinea-pig at that time and the heart blood, which yielded a pure culture of a typical pneumococcus, was put away in a sealed pipette, in the dark, at room temperature for over five years. At this time a culture on blood agar yielded a pure growth of markedly hemolyzing colonies. Smears showed gram-staining diplococci and short chains. This strain as a hemolytic streptococcus had a marked affinity for joints. By growth in symbiosis with *B. subtilis* on blood agar it acquired the cultural and pathogenic features of *Str. viridans*, producing repeatedly endocarditis without arthritis. After this modified strain had been passed through 17 rabbits (intravenous injections) it resembled the green-producing strains from rheumatism and produced non-suppurative arthritis, endocarditis, and pericarditis. The eighteenth passage produced in addition a few muscle lesions, appendicitis, and a myocarditis. After 21 animal passages (the last three through guinea-pigs by intraperitoneal injection) the affinity for joints, endocardium, myocardium, and muscles was lost, and it now produced pulmonary hemorrhages when injected intravenously in rabbits, and death from pneumococcemia when injected intraperitoneally and subcutaneously. It was now a pneumococcus in every respect.

Strain R51A was isolated originally October, 1902, as a pneumococcus from the blood during life in a case of lobar pneumonia. It was passed through 51 guinea-pigs at that time and was cultivated on blood agar in sealed tubes until the fall of 1912. During this time it always produced green on blood agar. Three attempts to restore its virulence were unsuccessful, but two years ago it was found to have acquired a moderate degree of virulence for mice, rabbits, and guinea-pigs, altho an attempt to raise its virulence to a high point failed. After three generations in unsealed tubes of blood agar, the last of which had become very dry, the strain had acquired the power, for the first time in over 10 years, to hemolyze blood. A subculture from a tube which had been kept sealed still showed green non-hemolyzing colonies. Neither of these colonies fermented inulin. The hemolyzing strain was now passed through 16 rabbits (intravenous injection), and the joint culture from the sixteenth animal showed both green and hemolyzing colonies. By intraperitoneal injection of the green-producing strain successively in five guinea-pigs it acquired a capsule, the power to ferment inulin, to produce acid in pneumonic serum, and to dissolve in bile.

Strain T was isolated as *Str. mucosus* from the pus in a case of acute mastoiditis three years ago. It was passed through two guinea-pigs at that time and cultivated, with frequent transfers, for a number of months. A subculture was then made on blood agar, the tube sealed with a paraffin cork, incubated for a number of weeks and put at room temperature in the dark until two weeks ago. Subcultures now yielded an organism which resembled *Str. viridans* in every respect and on intravenous injection in rabbits it did not grow in the blood but produced endocarditis in one rabbit after three injections.

THE EFFECT OF GROWTH IN A HIGH OXYGEN PRESSURE.

The results obtained in an experiment begun on January 13, 1913, will serve to illustrate the results obtained by growing hemolytic streptococci in an atmosphere of pure oxygen. Sixteen strains of hemolytic streptococci, isolated originally from a wide range of sources, which always yielded only hemolyzing colonies,

were selected. Cultures were made on the surface of small tins, 2 cm. in diameter, containing ascites-dextrose agar. These were now placed in a sterilized five-gallon bottle. The air was displaced with oxygen by means of a tube which extended to the bottom, the bottle sealed and placed at 37° C. for 72 hours. The oxygen was washed in water and passed through a long plug of sterilized cotton in a sterile pipette. Subcultures were made in the same way on January 17 and 22. On January 25 blood agar plate cultures were made. All but seven of the strains died. Two of these yielded grayish green colonies only, none of which was adherent; three produced non-adherent gray, adherent green, as well as hemolyzing colonies, while two of the strains yielded only hemolyzing colonies. The zone of hemolysis in each, however, was no longer wide and clear but narrow and hazy. The organisms in the control cultures in tubes of ascites-dextrose agar to which no oxygen was added remained unaltered. All yielded only widely hemolyzing colonies.

Seven strains of pneumococci were treated in the same way. All lost their capsule and virulence. Three were changed so that they lost the power to produce green, and acquired hemolyzing power. All of these and two others lost the power to ferment inulin. The hemolyzing strains all produced clouding in ascites-dextrose agar. Two now produced small non-adherent colonies which did not affect the media perceptibly. Smears showed chiefly small cocci which were often in clumps and an occasional short chain. The effect of oxygenated blood and methemoglobin on pneumococci will be discussed later. In this connection the observation was made that pneumococci grow better and live longer when subject to a high oxygen pressure than hemolytic streptococci.

Three strains of *Str. viridans* were treated in the same way. One changed into the hemolyzing variety; the other two were not changed perceptibly.

THE EFFECT OF GROWTH IN SYMBIOSIS WITH OTHER BACTERIA.

Twelve of 17 strains of hemolytic streptococci were converted into *Str. viridans* by growth in symbiosis with *B. subtilis* on blood agar. Five recently isolated strains remained quite unchanged,

but after three of these strains had grown in oxygen where their hemolyzing power was reduced, on being again grown in symbiosis with *B. subtilis*, they took on cultural and other features of *Str. viridans*. Growth in broth in symbiosis with *B. subtilis* resulted positively in only one instance. Pneumococci and *Str. viridans* remain quite unchanged when grown in symbiosis with this organism. Two strains of typical pneumococci, one representing a "pure line," were changed into hemolytic streptococci by growth in symbiosis on blood agar with a hemolytic colon bacillus.

THE EFFECT OF GROWTH IN HYPOTONIC AND HYPERTONIC MEDIA.

The effect of distilled water, of ovomucoid, hypotonic and hypertonic broth, and dialyzed beef serum agar was studied on eight strains of hemolytic streptococci, five strains of pneumococci, and one strain each of streptococcus from rheumatism and *Str. viridans*.

Salt-free broth was prepared and to this varying concentrations of Ringer's solution were added. Two strains of hemolytic streptococci lost their hemolyzing power completely in hypotonic media and produced green and gray colonies. Two strains only were perceptibly modified in hypertonic media (10 and 20 times the concentration of a normal Ringer's solution). In both, the colonies on blood agar plates were extremely small but surrounded by a wide, perfectly clear zone of hemolysis. Smears of these showed small cocci and chains, similar to certain strains of streptococci isolated at times from crypts of tonsils. All the strains in distilled water and hypotonic media remained unaltered. After growth in broth made 10 times the strength of a normal Ringer's solution one strain showed a zone of hemolysis peripheral to a green colony in which corpuscles were intact, and when the concentration of salt was twice as great as this there was a clean-cut hemolysis. The strain from rheumatism was changed from a green-producer to a hemolyzer in distilled water. The *Str. viridans* remained unaltered. The effect of ovomucoid media on pneumococci is often quite striking. Typical strains are frequently so altered that instead of producing green colonies on blood agar they produce rather large gray colonies, smears showing micrococci, and only occasionally short chains which are made up of round cocci. The

TABLE 3.

FERMENTATIVE AND OTHER PROPERTIES OF VARIOUS STRAINS AS HEMOLYTIC STREPTOCOCCI AND AS *Str. viridans*.

MEDIA	STRAINS													
	L		I		713		R51A		319		683		736	
	As Hem. Str.	As Str. Vir.	As Hem. Str.	As Str. Vir.	As Hem. Str.	As Str. Vir.	As Hem. Str.	As Str. Vir.	As Hem. Str.	As Str. Vir.	As Hem. Str.	As Str. Vir.	As Hem. Str.	As Str. Vir.
Blood agar	H	G	H	G	H	G	H	G	H	G	H	G	H	G
Serum-dextrose agar. Aerobic	++	+	+	++	+	o	++	o	+	o	+	+	+	+
Serum-dextrose agar. Anaerobic	+++	++	++	+++	+++	+	+++	+	++	++	++	++	+++	++
Inulin	o	+	o	+	o	+	o	+	o	+	o	o	o	o
Pneumonic serum	o	o	−	−	o	++	o	++	o	+	−	−	o	o
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	++	+
Maltose	o	+	+	+	+	+	+	+	+	+	o	+	+	+
Saccharose	o	+	+	o	+	+	+	+	+	+	+	o	+	+
Lactose	+	+	+	o	+	o	+	+	+	+	+	+	+	+
Mannite	o	o	o	o	+	o	+	+	o	o	+	o	+	o

In Table 3 Strains L and I were originally isolated as hemolytic streptococci, Strains 713, R51A, 319, and 683 as pneumococci, and Strain 736 from a joint in rheumatism.

fermentative and other properties are also markedly changed. The morphological and other features at times resemble staphylococcus so much that controls must be made to rule out contaminations.

FERMENTATIVE POWERS OF VARIOUS STREPTOCOCCI.

Table 3 shows that when strains of hemolytic streptococci have been converted into *Str. viridans* and pneumococci into hemolytic streptococci and *Str. viridans* the modified strain in each case has acquired new fermentative powers or has lost the power to ferment one or more sugars or both. It shows further the value of blood agar media, which have been used throughout this study, as a criterion of change in the various strains. Human blood was used. Rabbit and guinea-pig blood may be used in a study of this kind, but goat and sheep blood, while they serve to differentiate typical hemolytic streptococci and pneumococci, fail to bring out characteristic differences of the intermediate strains. Dog blood is practically useless in the differentiation of these organisms because the corpuscles hemolyze readily and spontaneously.

THE EFFECT OF FILTRATES OF ASCITES BROTH CULTURES OF VARIOUS STRAINS OF STREPTOCOCCI ON THEIR GROWTH (MARMOREK'S TEST).

The organisms were grown in ascites plain broth for 48 hours, the broth filtered through Chamberland filters, cultures made to test their sterility, and the strains as indicated in Table 4 inoculated. The strain of hemolytic streptococcus (L) was isolated in pure culture from the pus in a case of long-standing empyema; the pneumococcus from the blood in lobar pneumonia; *Str. viridans* (No. 722) from the blood during life in a case of subacute infectious endocarditis, and the rheumatic strain from the joint in a case of rheumatism.

Table 4 shows that the strains of streptococci failed to grow in the streptococcus filtrates but grew in filtrates of cultures of pneumococcus, the viridans, and rheumatic streptococci. The two strains of pneumococci and the rheumatic strain failed to grow in pneumococcus filtrate but grew well in filtrates of cultures of hemolytic streptococcus, viridans, and the rheumatic streptococcus. The *Str. viridans* grew in filtrates of hemolytic streptococcus, of all but one pneumococcus, and of the rheumatic streptococcus.

The rheumatic strain (No. 736) which produced a slight hemolysis failed to grow in streptococcus filtrates while one which produced green did. This has been found to be the case with other strains.

TABLE 4.

THE EFFECT OF ASCITES BROTH CULTURE FILTRATES OF HEMOLYTIC STREPTOCOCCI, PNEUMOCOCCI, "STR. RHEUMATICUS," AND *Str. viridans* ON THE GROWTH OF THESE ORGANISMS.

FILTRATES	STRAIN INOCULATED									
	Streptococcus L	Pneumococcus S	Pneumococcus 678[19]	XII[2] As a Streptococcus	XII[2] As a Pneumococcus	595 As a Streptococcus	595 As a Pneumococcus	"Str. rheumaticus" 736	734 As a Streptococcus	734 As a Pneumococcus	722
Hemolytic streptococcus (L)	o	+	+	o	+	o	+	o	+	+	+
Pneumococcus	++	o	o	++	o	++	o	++	o	o	++
Strain XII[2] as a pneumococcus	++	o	o	++	o	++	o	++	o	o	++
Strain 595 as a pneumococcus	+	o	o	++	o	++	o	++	o	o	o
Str. viridans (722)	++	+	+	++	++	++	++	++	++	++	o
"Str. rheumaticus" (734)	++	+	+	++	++	++	++	++	o	++	++
Ascites-plain broth (control)	+	o	+	+	+	+	o	+	+	+	+

It is thus seen that the strains made over into pneumococci behave as do other strains of pneumococci toward filtrates of hemolytic streptococci and of pneumococci.

SUMMARY OF CHARACTERISTICS OF VARIOUS STRAINS OF STREPTOCOCCI AND PNEUMOCOCCI.

In Table 5 is given the morphology and the behavior of various strains which were originally either hemolytic streptococci, "*Str. rheumaticus*" or pneumococci, toward the various tests used in differentiating these organisms. These tests were made after the transformation of one into the others was seemingly complete. During the transition stages the cultural tests fail to give characteristic reactions and it is often difficult to know where an organism belongs, but after the organisms, which have the morphology, cultural, and pathogenic properties of hemolytic streptococci, have been converted into *Str. viridans* and then have acquired a capsule and high virulence from animal passage, they react like pneumococci in every respect. In order to satisfy myself further that the streptococci which I had converted into pneumococci were really streptococci and those transformed really pneumococci,

TABLE 5.

SUMMARY OF THE MORPHOLOGY, CULTURAL AND OTHER PROPERTIES OF VARIOUS STRAINS AS STREPTOCOCCI AND AS PNEUMOCOCCI.

STRAIN	MORPHOLOGY, CULTURAL AND OTHER PROPERTIES													
	As a Streptococcus							As a Pneumococcus						
	Morphology	Blood Agar Plates	Clouding of Serum Dextrose Agar	Fermentation of Inulin Broth	Acid in Pneumonic Serum	Solubility in Bile	Autolysis in NaCl Solution	Morphology	Blood Agar Plates	Clouding of Serum Dextrose Agar	Fermentation of Inulin Broth	Acid in Pneumonic Serum	Solubility in Bile	Autolysis in NaCl Solution
595	Diplococci and short chains	H	++	o	o	o	o	Encapsulated, often lance-shaped diplococci	G	o	++	++	+	+
XII[2]	Diplococci and rather long chains	H	+	o	o	o	o	Encapsulated diplococci and short chains	G	o	+	++	++	+
735	Diplococci and short chains	H	+++	o	o	o	o	Widely encapsulated diplococci	G	o	+	+	++	+
736	Diplococci and short chains	H	+++	o	o	o	o	Encapsulated, often lanceolate diplococci	G	o	++	++	+	+
319	Diplococci, short chains, and small clumps	H	+	o	o	o	o	Encapsulated, often lanceolate diplococci	G	o	+	++	+	+
R51A	Diplococci and chains	H	++	o	o	o	o	Encapsulated, often lanceolate diplococci	G	o	++	+	++	+
734	Diplococci and short chains	H	+++	o	o	o	o	Encapsulated diplococci	G	o	++	++	+	+
678	Diplococci and short chains*	H	++	o	o	o	o	Encapsulated, often lance-shaped diplococci	G	o	++	+	+	+
2.18[17]	Diplococci and chains	H	++	o	o	o	o	Encapsulated, lanceolate diplococci	G	o	+	++	++	+

*With the exception of Strain 678, there were no capsules

I felt that their identification by an independent observer was highly desirable. Through the kindness of Dr. Libman I was able to do this. A series of 10 cultures containing known hemolytic streptococci, *Str. viridans*, and pneumococci, together with strains as streptococci and as pneumococci, was sent to him as unidentified cultures. They were studied in the laboratories of Mt. Sinai Hospital, New York, and Dr. Libman's identification agreed with mine in every respect.

EXPERIMENTS ON AGGLUTINATION.

Cole and his co-workers would divide pneumococci into four groups according to the reactions of agglutination and the specific protection of the immune serum.[1] As this division appears to hold good for pneumococci as they occur in pneumonia it no doubt marks an advance in the efforts to secure effective antipneumococcal serum. Specific sera have been secured by Cole and his associates for Groups I and II which contain the types of pneumococci most frequently encountered in pneumonia, the cases caused by Group II being especially severe and associated with a moist pneumonic exudate.

In view of these considerations the results of observations on a pneumococcus culture, seemingly of Group I, are of interest.

Pneumococcus 678 was isolated from the blood of a pneumonia patient October 25, 1912, and after two transplantations on blood agar a culture was secured from a single coccus by Dr. V. H. Moon. The colonies of this pure line were typically green; the organism fermented inulin, produced acid in pneumonic serum, dissolved readily in salt solution and in bile and sodium oleate. Growth continued typical of pneumococcus on blood agar in closed tubes, but the capsule was lost. After passage through 22 guinea-pigs, the virulence, the capsule, and the growth increased. Spreading colonies developed on blood agar plates similar in every way to the colonies from pneumonic lungs with a slimy exudate.

Inoculations (before the guinea-pig passage) on ascites-dextrose agar in pure oxygen resulted after three transplantations in loss of virulence and at the same time in a gain of hemolytic power. After passing through 15 rabbits, the injections being intravenous, the organisms of this strain continued hemolytic, were not affected by salt solution or bile, and did not ferment inulin nor produce acid in pneumonic serum.

On June 24, 1913, the original strain that remained as a pneumococcus, and the strain after animal passage, produced green colonies only on blood agar; and the hemolyzing strains produced only colonies with typical zones of hemolysis. Agglutination tests of these various strains were now made with Dr. Cole's Sera I and II (Table 6).

[1] *Jour. Am. Med. Assn.*, 1913, 51, p. 663; also Dochez and Gillespie, *ibid*, p. 727.

TABLE 6.

VARIATIONS IN AGGLUTINATION OF STRAIN 678 AS PNEUMOCOCCUS AND AS STREPTOCOCCUS.

SERUM	AS PNEUMOCOCCUS		AS STREPTOCOCCUS	
	Before Passage	After Passage	Before Passage	After Passage
I	+	o	o	o
II	o	++	o	o

For Antipneumococcus Sera I and II, I am indebted to Dr. R. I. Cole, Director of the Hospital of the Rockefeller Institute for Medical Research, New York. These sera have strong agglutinating powers.

The mixtures were made by adding 0.5 c.c. of serum to 0.2 c.c. of the washed suspension of cocci in salt solution (in each case approximately one-sixth of a blood agar slant). The tubes were incubated at 37° C. for two hours and then placed at room temperature.

We have here a pneumococcus which would be classed in Group I when isolated from the blood of the pneumonia patient, but after passing through guinea-pigs, the agglutination places in Group II, while the streptococcus mutations practically are not agglutinated at all.

A similar experiment made on a large scale by using six different cultures gave the results recorded in Table 7. Two of the cultures, Strains 595 and XII[2], were originally hemolyzing streptococci; two, Strains 734H and 736, were isolated from the joints in rheumatism; and two, Strains 319 and 678, were originally pneumococci.

TABLE 7.

AGGLUTINATION TESTS OF VARIOUS STRAINS AS HEMOLYTIC STREPTOCOCCI AND AS PNEUMOCOCCI.

SERUM	595		XII[2]		734H		319		736		678	
	As Streptococcus	As Pneumococcus	As Streptococcus	As Pneumococcus	As Streptococcus	As Pneumococcus	As Streptococcus	As Pneumococcus	As Streptococcus	As Pneumococcus	As Streptococcus	As Pneumococcus
Antipneumococcus I	o	o	o	o	o	o	o	++	o	o	o	+
Antipneumococcus II	o	++	o	++	o	++	o	+	o	+	o	+
Antipneumococcus M	o	+	o	+	o	+	o	+	o	+	o	+
Antistreptococcus M	+	o	+	o	o	o	+	o	+	o	+	o

Antipneumococcus Serum M and Antistreptococcus Serum M were obtained from Mulford and Co. through the courtesy of Dr. Hitchens.

We see that as streptococci they are with one exception agglutinated by antistreptococcus serum but not by antipneumococcus serum, while as pneumococci they are agglutinated by antipneumococcus serum only and with one exception most strongly by Antipneumococcus Serum II. The strains which have all the other

features of streptococci behave as streptococcci and the strains which are like pneumococci in other respects behave like pneumococci with respect to agglutinating serum.

That pneumococci easily are made to change their agglutinative properties is shown also by some experiments I made with cultures of Pneumococcus, Groups I and II, which Dr. Cole kindly sent me. After growing the organisms on dextrose blood agar, ascites-dextrose agar and broth, and in ascites broth and washing them in NaCl solution I found that only a degree of specific agglutination occurred. Pneumococcus I was agglutinated by Serum I when grown in all media except ascites-dextrose agar; the organisms from this medium were agglutinated by Serum II. Pneumococcus II was agglutinated specifically when grown in ascites-dextrose broth; it was agglutinated by Sera I and II when grown on the other media.

These results are in accord with those of Bordet and Sleeswijk[1] and Gay and Claypole,[2] the former demonstrating variations in the agglutinability of the bacillus of whooping cough, the latter inducing variations in the agglutinability of the bacillus of typhoid fever.

TRANSFORMATION OF PNEUMOCOCCUS GROUPS I AND II INTO STREPTOCOCCI.

Through the kindness of Dr. Cole I have been enabled to experiment with his Pneumococci I and II, the cultures used by Dr. Cole and his associates to develop Antipneumococcus Sera I and II. Pneumococcus I was isolated originally by Professor Dr. Neufeld.[3]

In order to subject the possibility of transformation of pneumococci into streptococci to a supreme test I therefore subjected cultures of Pneumococci I and II to a wide range of conditions. Altogether Pneumococcus I has been passed through 70 animals, Pneumococcus II through 16. Both were highly virulent and resistant to phagocytosis, and corresponded to typical pneumococci in every respect. Pneumococcus II, however, produced the

[1] *Ann de l'Inst. Pasteur*, 1910, 24, p. 476.

[2] *Jour Am. Med. Assn.*, 1913, 60, p. 1141.

[3] Neufeld and Haendel, *Arb. a. d. k. Gsndhtsamte*, 1910, 24, p. 293.

moister growth and its capsule was distinctly wider and more easily stained.

As pointed out it is difficult to produce mutations of highly virulent pneumococci because they usually die before important changes take place. After suspending growths of Pneumococci I and II in hypertonic Ringer's solution, in distilled water, and after cultivation in ovomucoid media, in sterilized urine, in plain broth, in broth to which sodium iodbenzoate and sodium iodoxybenzoate had been added, clean-cut hemolyzing colonies were not obtained on blood agar altho in some colonies hemolysis was separated from the colony itself by a zone in which the corpuscles were intact. Blood agar plates were inoculated every day at first and then every other day until the tubes became sterile.

A medium in which virulent pneumococci live longer when subjected to changes in oxygen pressure was found in sterile defibrinated blood. The oxygen tension could be made high or low as desired by shaking with oxygen or carbon monoxid gas.

On July 11, Pneumococci I and II and two other virulent pneumococci (Nos. 678 and 734) were inoculated each into two eight-ounce bottles containing 2 c.c. of sterile human blood. In each case the air in one bottle was displaced by oxygen, in the other by illuminating gas (carbonic oxid). The gases were washed and passed into the bottles through long well-fitting cotton plugs in sterile pipettes, the bottles tightly closed with rubber stoppers and sealed with paraffin. They were now placed in a shaker at 37° C. for 24 hours. Inoculations were now made from the bottles which were put in diffuse sunlight at room temperature until July 20, inoculations being made on blood agar plates in 3, 5, 8, and 14 days. Pure cultures of green colonies in diminishing numbers were obtained in each instance until the fourteenth day, the number of visible organisms per loop now having dropped to under 100 in each of the bottles containing oxygen whereas in those containing the gas the number was higher. Pneumococcus II and Pneumococcus 678 in the bottles containing oxygen gave a number of fine colonies surrounded by hemolysis, but the hemolysis was outside of a zone in which the corpuscles were intact. On July 30 one of these bottles gave 11, and the other, five colonies which produced a clear zone of hemolysis that began immediately outside the colony itself. At the same time the oxygen bottle containing Pneumococcus II gave nine green colonies, and the oxygen bottle with Pneumococcus 678 gave 53.

Pneumococcus I was lost in the oxygen treated blood, while from the blood saturated with carbonic oxid was obtained a number of colonies, none showing any noteworthy change.

Inoculations on blood agar plates from four of the hemolyzing colonies of Pneumococcus II and from two of Strain 678 yielded only hemolyzing colonies.

Two similar experiments, in which different samples of blood were used, were made on August 6 and 15. Strains 734H, 736, and XII, which had been made into

pneumococci, were included. In only two instances were hemolyzing colonies obtained from the blood saturated with carbonic oxid. Two of the mutant strains, Nos. 736 and XII, yielded hemolyzing colonies from the oxygen treated blood in both experiments; the other, Strain 734H, in only one. Pneumococcus II yielded hemolyzing colonies again in the experiment begun August 15 but not in the one begun August 6. Pneumococcus I proved most refractory, but yielded seven hemolyzing colonies on the thirteenth day in the experiment begun August 15 together with 23 green colonies. Hemolyzing colonies were obtained also on the following day. Plate inoculations from four of these colonies yielded hemolyzing colonies only.

The results of further tests with the original and modified organisms are given in Table 8.

TABLE 8.

CHARACTERISTICS OF PNEUMOCOCCI I AND II AS PNEUMOCOCCI AND AS STREPTOCOCCI.

STRAIN	CAPSULE	AGGLUTINATION BY ANTIPNEUMOCOCCUS SERUM	SUSCEPTIBILITY TO PHAGOCYTOSIS	ACID IN PNEUMONIC SERUM	CLOUDING OF ASCITES-DEXTROSE AGAR	FERMENTATION OF CARBOHYDRATES				AUTOLYSIS IN NaCl SOLUTION	SOLUBLE IN BILE
						Inulin	Mannite	Raffinose	Saccharose		
I as Pneumococcus . . .	+	+	o	+	o	+	o	++	+	+	++
I as Streptococcus . . .	o	o	+	o	++	o	++	o	+	o	o
II as Pneumococcus . .	+	+	o	+	o	+	o	+	+	+	++
II as Streptococcus . .	o	o	+	o	+	o	+	o	+	o	o

The difference in the behavior of the organisms when treated with bile was striking. The growth from blood agar was washed once in NaCl solution and bile then added. The pneumococcal form of the organisms dissolved in 15 minutes whereas the streptococcal forms remained gram-positive for 48 hours.

The agglutination reactions of various cultures of Pneumococci I and II were now determined. The results are given in Table 9.

A slight clumping of the streptococcal forms was observed at the end of 24 hours in Antipneumococcus Sera I and II. A diffuse turbidity, however, was still present and the sediment easily broken up by shaking. Similar sediments were obtained in the case of organisms grown continuously on blood agar when Pneumococcus I was acted on by Serum II and Pneumococcus II by Serum I. This was not considered a true agglutination, but to satisfy myself further on this point the effect of Sera I and II was tested on five typical strains of hemolytic streptococci. The clumping of the streptococci at the end of 24 hours was comparable

to that of I and II as streptococci. In Table 7, Strains 595, XII, 734H, and 736 as pneumococci were agglutinated most strongly by Serum II; after treatment in highly oxygenated blood in which four of the strains yielded hemolyzing colonies, which were no longer

TABLE 9.

AGGLUTINATION OF PNEUMOCOCCI I AND II AS PNEUMOCOCCI AND AS STREPTOCOCCI.

STRAIN	SERUM							
	Antipneumococcus Serum I		Antipneumococcus Serum II		Antipneumococcus Serum M		Antistreptococcus Serum M	
	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.
I Cultivated continuously on blood agar	Complete	Solid coagulum	0	0	0	Loose coagulum	0	0
II Cultivated continuously on blood agar	0	0	Complete	Solid coagulum	0	Loose coagulum	0	0
I While in oxygen but still a pneumococcus	Complete	Coagulum less firm	Beginning	Complete	0	±	0	0
II As a streptococcus	0	0*	0	0*	0	0	Beginning	Complete
II While in oxygen but still a pneumococcus	Beginning	Complete	Marked	Solid coagulum	0	±	0	0
II As a streptococcus	0	0*	0	0*	0	0	0	Complete

* See statement in text, p. 25.

Same technic as in experiment recorded in Table 6.

agglutinated by antipneumococcus serum, two of the strains (Nos. 595 and 736), still growing as pneumococci, were agglutinated by Serum I instead of Serum II while the other two were agglutinated equally well by both.

In Table 10 are given the results of the agglutination by various dilutions of Serum I of Pneumococci I and II as pneumococci and as streptococci. The result is clear and needs no discussion. The mixtures were kept at 37° C. for five hours, then at room temperature.

The effect of the organisms on rabbits and mice was next studied. The original cultures killed mice after intraperitoneal injection, and rabbits after intravenous injections, with a rapid pneumococcemia. On the other hand the mice injected with comparable doses of the streptococcal modification recovered, and peritoneal smears 5 and 15 hours after injection showed marked

phagocytosis. Rabbits were injected with each of the streptococcal forms and also with comparable doses (one-fourth blood agar slant) of the original pneumococcus cultures. The two injected with the pneumococcus culture died in 24 hours with pneumococcemia; both showed marked pulmonary hemorrhages, hemorrhagic enteritis and acute splenitis, the joint fluid remaining clear. The rabbits injected with the streptococcal forms remained

TABLE 10.

THE EFFECT OF DILUTING SERUM I ON ITS AGGLUTINATING POWER OVER PNEUMOCOCCI I AND II AS PNEUMOCOCCI AND AS STREPTOCOCCI.

STRAIN	AGGLUTINATING POWER OF VARIOUS DILUTIONS OF SERUM I											
	1–10			1–30			1–90			1–270		
	1 hr.	5 hr.	48 hr.	1 hr.	5 hr.	48 hr.	1 hr.	5 hr.	48 hr.	1 hr.	5 hr.	48 hr.
I As a pneumococcus....	+	++	++	+	+	++	o	Beginning	++	o	o	o
I As a streptococcus	o	o	Slight	o	o	Slight	o	o	o	o	o	o
II As a pneumococcus...	o	Slight	Solid coagulum	o	o	+	o	o	o	o	o	o
II As a streptococcus....	o	o	Slight	o	o	o	o	o	o	o	o	o

apparently well after the first injection, but succumbed in from two to five days after an additional and larger injection. In both, the joint fluid was markedly turbid and contained hemolyzing streptococci. One rabbit had an "ascending" nephritis, and one a few muscle lesions; neither had endocarditis nor pericarditis. To study further the changed pathogenic power, on August 15 two rabbits were injected with larger doses of the streptococcal growths. The record of one of these rabbits is given.

August 16. Rabbit seemed quite well; knee joints punctured. Turbid fluid with leukocytes and diplococci. Cultures of joint fluids and blood on blood agar plates.

August 17. Blood sterile; joint fluids showed large number of hemolyzing colonies in pure culture.

August 19. Lame in right hind leg. Knee joint swollen and tender.

August 21. Found dead. Cloudy swelling of heart and kidneys, no distinct focus of infection in the kidney; acute cholocystitis; gall bladder distended with a bile-stained mucus in which were flakes of leukocytes and many diplococci and streptococci; in the wall of the gall bladder were a number of small, grayish nodules which

projected on the inner surface and involved chiefly the mucous membrane. On transmitted light it was found that these nodules occurred in the parts farthest removed from the blood vessels. Smears from these areas, the tissue of which was washed and then crushed, showed leukocytes and gram-staining diplococci in short chains. Paraffin sections of the gall bladder showed streptococci in these areas. Right knee joint distended with turbid, thick fluid, with many leukocytes, endothelial cells, and a few diplococci. One small streak in the subscapular muscle. Stomach, appendix, liver, and spleen normal. Cultures on blood agar plates.

August 23. Cultures; blood sterile; crushed tissue from gall bladder and bile gave large number of hemolyzing colonies only; joint fluids yielded three green and no hemolyzing colonies; pelvis of kidney, a few hemolyzing colonies.

It is thus shown that as the morphology, cultural, and agglutinating properties of the two strains change, the pathogenic properties change likewise. Just as in the strains isolated by me they act in every way like certain strains of hemolytic streptococci. From these results it appears that while pneumococci under certain conditions may be divided into distinct groups by means of agglutination and other reactions, a change of environment may produce radical changes in agglutinative as well as other properties. The fact that they may even be converted into streptococci is, nevertheless, no reason why varieties of pneumococci may not be found in pneumonia, for example, as worked out by Cole and his associates, but to consider the varieties as "fixed" races hardly seems warranted.

SUMMARY.

Altogether 21 strains isolated originally as hemolytic streptococci from a wide range of sources, including erysipelas, scarlet fever, puerperal sepsis, arthritis, tonsillitis, cows' milk, etc., have in one way or another been converted into *Str. viridans;* 3 into *Str. viridans* and into typical pneumococci, and 1 into *Str. mucosus* as well; one of these corresponded at one time to the streptococci from rheumatism.

Seventeen strains which were isolated as *Str. viridans* chiefly from the blood and tonsils in cases of chronic infectious endocarditis and two strains from cows' milk have been converted into pneumococci and 2 of these into *Str. mucosus* also; 10 have been made to take on cultural and morphologic characteristics of hemolytic streptococci, in 2 of which the pathogenic powers were

shown to be those of hemolytic streptococci; 1 strain was converted into hemolytic streptococcus, into *Str. viridans*, and into a pneumococcus.

Eleven strains isolated as pneumococci from the sputum, blood, and lung in pneumonia, and from empyema and Cole's Strains I and II have been made to correspond to hemolytic streptococci; 7 took on the features of *Str. viridans;* the streptococci from 3 of these strains by animal passage acquired all the essential features of the streptococci of rheumatism; 2 have been converted into hemolytic streptococcus, the streptococci of rheumatism, *Str. viridans*, and back again into pneumococcus.

Five strains of *Str. mucosus* have taken on the cultural features of hemolytic streptococci. Two of these were converted into *Str. viridans.*

Five strains of the streptococcus of rheumatism have taken on the features of hemolytic streptococci, 2 of *Str. viridans*, and 4 have been converted into pneumococci.

In order to meet the objection that even tho every ordinary precaution was taken to obtain pure cultures, I was working with mixtures whenever mutation was observed, cultures of each main variety were obtained from single organisms by the Barber method. The same results were obtained with three of these "pure line" cultures of hemolytic streptococci, 6 of *Str. viridans*, and 2 each of *Str. mucosus* and pneumococcus. Hence the changes observed are not due to mixtures nor to so-called "mass selection" but to actual changes wrought under the influence of changed environment.

The transformation of some of the strains has been found to be complete by every test known. Thus the morphology, the presence of capsule, the fermentative powers, the solubility or insolubility in bile and in NaCl solution, the behavior toward the respective broth culture filtrates (Marmorek's test), the specific immunity response, as manifest by the production of opsonin and agglutination by antipneumococcus and antistreptococcus serum, and the more or less specific pathogenic powers have been studied. Strains that corresponded to hemolytic streptococci have been converted into typical pneumococci as determined by all the above tests and vice versa.

Now that the various strains of the streptococcus group may be converted each one into the others the question of nomenclature comes up. For the present the names which have been used in the past must be continued because convenient and because it is at these points that they show certain distinctive morphological, cultural, and pathogenic properties.

The results obtained show clearly why the classification of streptococci based on fermentative powers alone has proven to be unsatisfactory.

The changes observed have frequently the characteristics of true mutations because they appear suddenly, under conditions more or less obscure and because the newly acquired properties persist unless the organisms are again placed under special conditions. A pre-mutational stage seems to be necessary because the same strain will not yield mutants when placed under what seem to be identical conditions at different times. The underlying conditions which tend most to call forth changes are, first, favorable conditions for luxuriant growth and then unfavorable conditions—under stress or strain. This seems to call forth new or latent energies which were previously not manifest and which now have gained the ascendency and tend to persist. This may hold true *in vivo* also. This fact makes it difficult to obtain mutations outside of the body with highly virulent strains, because they die before there is opportunity for the organisms to adjust themselves to the new conditions. It explains also why injection into cavities makes for greater changes than intravenous injections of moderately virulent organisms. Apparent mutations in animals have been observed almost exclusively in closed cavities, such as joints and pericardium, and here mostly when the tissues of the host were gradually getting the upper hand and the organisms were being destroyed. The mutations *in vitro* may be spoken of as "retrogressive" and those observed in animals as "progressive" because in the former virulence, fermentative powers, and other evidences of a vigorous vegetative life are diminished whereas in the latter they are usually increased.

The bearing these results have on bacteriology, epidemiology, and medicine might be discussed at length; only the following

point will be mentioned: the fact that variations in oxygen tensions, and salt concentration, that growth in symbiosis with other bacteria and that injections into cavities in animals commonly call forth mutational forms in streptococci suggests strongly that similar changes might occur in various foci of infection where such conditions may prevail. It would seem, therefore, that focal infections are no longer to be looked upon merely as a place of entrance of bacteria but as a place where conditions are favorable for them to acquire the properties which give them a wide range of affinities for various structures.

From this study the apparent position of the various members of the streptococcus group may be illustrated by the position of the fingers in a partially flexed hand, in which hemolytic streptococcus occupies the position of the little finger, the pneumococcus the place of the index finger (the opposite extreme), *Str. viridans* (representing the group of more or less saprophytic, non-hemolyzing streptococci) the middle finger, the streptococci from rheumatism the fourth finger, and *Str. mucosus*, having some of the properties of both pneumococci and streptococci, the position of the thumb. In this grouping there is in general an increase in parasitism and virulence as we approach the thumb (*Str. mucosus*). Being members of the same family, the sign of reversible chemical reaction ($\rightleftarrows$) between each might be used to indicate their transmutability.

EXPLANATION OF PLATE 1.

The magnification of the microphotographs in each figure is 1,200 diameters. Rosenow's capsule stain was used. The apparently large size of the strains as pneumococci in the figures is due in a large measure to fixation and the amount of decolorization. When organisms are free from capsules as well as in those portions of the smear where the capsule is not stained, in strains which have capsules, they shrink during fixation with tannic acid. The larger the capsule the larger the organisms appear to be. This fact explains the differences and the apparently larger size of the pneumococci in Figs. 3, 6, and 7.

FIG. 1.—Strain 595 as a hemolytic streptococcus, isolated from a case of scarlet fever. Smear from 24-hour culture in ascites-dextrose broth. Gram stain.

FIG. 2.—Strain 595 as *Str. viridans*. Smear from 24-hour culture in ascites-dextrose broth. Gram stain.

FIG. 3.—Strain 595 as a pneumococcus. Smear from 24-hour culture in ascites-dextrose broth. Capsule stain.

FIG. 4.—Strain of streptococcus from rheumatism which produced slight hemolysis on blood agar and myositis in animals. Smear from blood agar slant. Capsule stain.

FIG. 5.—The same strain as Fig. 4 after it is transformed into a pneumococcus. Smear from blood agar slant. Capsule stain.

FIG. 6.—Highly virulent pneumococcus (Group I) isolated originally by Neufeld and sent to me by Dr. Cole. Smear from surface and water of condensation of blood agar slant. Capsule stain.

FIG. 7.—The same strain as in Fig. 6 after being transformed into a hemolytic streptococcus. Smears from the surface and water of condensation of blood agar slant. Capsule stain.

PLATE 1.

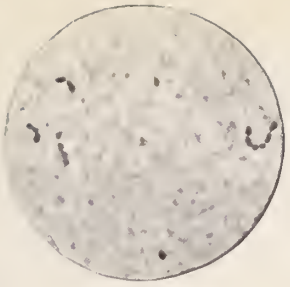

FIG. 1.

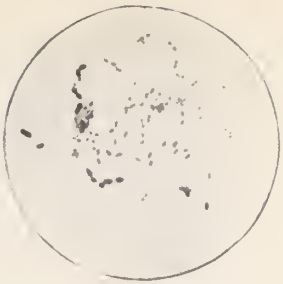

FIG. 2.

FIG. 3.

FIG. 4.

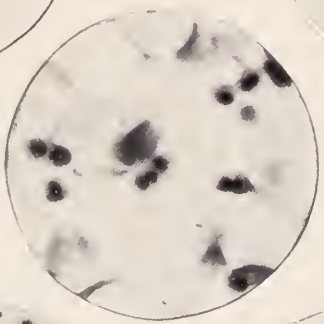

FIG. 5.

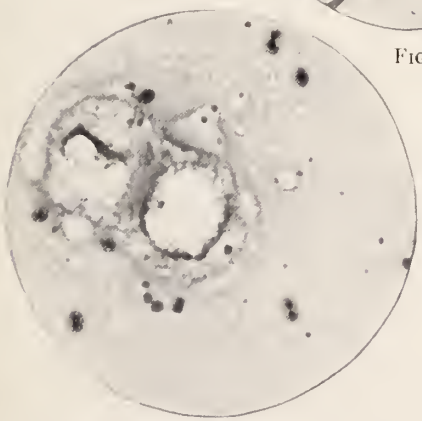

FIG. 6.

FIG. 7.

RABIES-HYDROPHOBIA.

A STUDY OF FIXED VIRUS, DETERMINATION OF THE M.L.D., VACCINE TREATMENT (HÖGYES, PASTEUR, AND DIALYZED VACCINE), AND IMMUNITY TESTS.*

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The object of this paper is to present, first, the results of research on fixed virus, i.e., the action of disinfectants, the effect of heat, passage through Berkefeld filters, infectivity, exposure to peritoneal fluid *in vivo*, and dialysis; second, the determination of the M.L.D. of the virus and the results of immunity tests on sets of animals which had been vaccinated by the various methods of antirabic treatment, i.e., Högyes, Pasteur, and a new method, devised in this laboratory, which will be designated the method of dialysis.

Throughout the experimental work, unless otherwise stated, a standard suspension (1–100) of fresh rabic brain in physiological salt solution was employed. The preparation of this standard homogeneous suspension is given in detail under the consideration of the M.L.D.

The following tabulations show the effect of disinfectants on the virus. Their action was determined by injecting 0.5 c.c. of the test suspension intracranially into either rabbits or guinea-pigs. At the termination of each series of injections from the test suspensions the original untreated suspension was injected as a control.

EXPERIMENT 1.

	Exposure	Death from Rabies
Effect of 1 per cent tricresol on virus suspension	5 hours	6th day
	7 "	" "
Effect of 1 per cent phenol on virus suspension	5 "	" "
	6 "	" "
Effect of 0.4 per cent formaldehyde on virus suspension	5 "	Lived[1]
	6 "	"
Control		6th day

EXPERIMENT 2.

	Exposure	Death from Rabies
Effect of 0.04 per cent formaldehyde on virus suspension	15 minutes	6th day
	30 "	" "
	1 hour	" "
	1.5 hours	7th "
	2 "	8th "
Control		7th "

[1] Throughout this paper the word "lived" indicates that the animal was alive two months after the injection.

* Received for publication August 12, 1913.

EXPERIMENT 3.

	Exposure	Death from Rabies
Effect of 0.08 per cent formaldehyde on virus suspension	1 hour	6th day
	1.5 hours	7th "
	2 "	Lived
	2.5 "	"
Control		6th day

EXPERIMENT 4.

	Exposure	Death from Rabies
Effect of 0.1 per cent formaldehyde on virus suspension	1 hour	6th day
	2 hours	Lived
	3 "	"

EXPERIMENT 5.

	Exposure	Death from Rabies
Effect of 0.5 per cent acetone on virus suspension	3 hours	6th day
	6 "	" "
Effect of 0.5 per cent acetaldehyd on virus suspension	3 "	Lived
	6 "	"
Effect of 0.5 per cent benzaldehyd on virus suspension	3 "	"
	6 "	"

	Exposure	Death from Rabies
Effect of 0.5 per cent furfurol on virus suspension	3 hours	Lived
	6 "	"
Effect of 0.5 per cent salicylaldehyd on virus suspension	3 "	6th day
	6 "	7th "
Effect of 0.5 per cent anisaldehyd on virus suspension	3 "	6th "
	6 "	" "
Control		" "

In the following experiment the rabic brain tissue was emulsified in distilled water instead of normal salt solution. The exposures were made at 37° C., while all others in this series were exposed at room temperature.

EXPERIMENT 6.

	Exposure	Death from Rabies
Effect of 2 per cent phenol on virus suspension	1 day	Lived
	2 days	"
Effect of 2 per cent sodium chlorid on virus suspension	1 day	6th day
	2 days	Lived
Effect of 0.4 per cent sodium chlorid and 0.4 per cent phenol on virus suspension	1 day	6th day
	2 days	7th "
	3 "	" "
	4 "	Lived

	Exposure	Death from Rabies
Effect of 0.85 per cent sodium chlorid on virus suspension	1 day	6th day
	2 days	8th "
	3 "	6th "
	4 "	7th "
	5 "	Lived

It will be noted that a one per cent phenol does not destroy the virus during an exposure of six hours, while a two per cent solution kills it in less than 24 hours. A two per cent solution of sodium chlorid has a similar effect to that of the two per cent phenol, in that it destroys the virus in less than two days. In the higher percentages of sodium chlorid or phenol the effect on the virus is to be considered as due to the nonisotonic condition of the suspension rather than to a disinfectant action. When a one per cent

suspension of fresh virus in a solution of sodium chlorid and phenol (0.4 per cent) is kept at 37° C. the virulence is not lost until the fourth day. This percentage of sodium chlorid and phenol will preserve the virulence of a five per cent fresh rabic brain, when kept in the cold room, for several weeks. On the other hand, most of the aldehyd compounds are extremely active in destroying the infectivity of fixed virus. A 0.5 per cent solution of acetaldehyd, benzaldehyd, formaldehyd or furfurol destroys the virus in less than three hours. A 0.5 per cent solution of salicylaldehyd, anisaldehyd or acetone has no effect up to an exposure of six hours. The specific disinfectant action of formaldehyd is shown by the fact that the virulence of the virus is lost when exposed for two hours to an 0.08 per cent solution. As a result of this work we advise, as a preliminary measure, the disinfection with formalin of all wounds made by animals suspected of being rabid.

The thermal death point of the virus is comparatively low; altho the results of heat tests vary somewhat with the same suspension under apparently the same conditions as to temperature and duration of exposure, yet these variations are not so great but that they fall within the range of experimental error. These experiments may be summed up as follows:

The virus is destroyed in 20 minutes at an exposure of 45° C.
" " " " " 15 " " " " 50° C.
" " " " " 5 " " " " 60° C.
" " " " " 2 " " " " 100° C.

Under certain conditions rabic virus readily passes through a tested Berkefeld filter, by a suction of 26 inches of mercury. This may be accomplished with great regularity if the rabic brain in suspension is not less than one per cent and is as nearly homogeneous as possible.

The accompanying table (p. 36) shows the results of two filtration experiments with the 1–25 suspension of rabic brain, one with the 1–70, and three with the 1–100 suspension.

It will be noted that the percentage of rabic brain in suspension influences the amount of virus in the filtrate. This is shown by determining the M.L.D. of the filtrate, and comparing this with the M.L.D. of the unfiltered suspension. For instance, the M.L.D.

of the unfiltered suspension is 0.5 c.c. of a 1–25,000 suspension of rabic brain, while the M.L.D. of the filtrate from the 1–25 suspension is represented by a dilution of approximately 1–1,000. This M.L.D. of the filtrate is one twenty-fifth that of the original suspension. Under similar conditions the M.L.D. of the filtrate from the 1–70 suspension is 0.5 c.c. of the 1–250 dilution. This dose represents one one-hundredth part of the virus in the unfiltered suspension. The undiluted filtrate from the 1–100 suspension was fatal in one out of three tests, and dilutions were not infectious.

Suspension of Rabic Brain	Filtrate Diluted to	Intracranial Injection of	Death from Rabies	Part of M.L.D.
1–25	1–800	0.5 c.c.	2 died	$\frac{1}{25}$
	1–1000	" "	1 " 1 lived	
	1–1200	" "	2 "	
1–70	1–200	" "	1 died	$\frac{1}{100}$
	1–250	" "	1 "	
	1–300	" "	1 lived	
1–100	1–100	" "	1 died 2 lived	$\frac{1}{250}$
	1–125	" "	3 "	
	1–150	" "	3 "	

When the undiluted filtrate is fatal the dose represents one two-hundred and fiftieth part of the virus in the unfiltered suspension. From these experiments it may be inferred: first, that rabic virus passes through a tested Berkefeld filter only under certain conditions as to homogeneity, dilution, and suction; second, that the amount of virus passing through the filter is inversely proportional to the dilution, other conditions being the same.

In considering the normal habitat of the virus, it is generally conceded that the mortality from inoculation depends upon the avenue of injection. For instance, in our experiments it was found that a single injection (1 c.c. of the standard suspension) into the lateral ear vein of a rabbit rarely infected. A series of these injections on three successive days usually terminated in death from rabies within four weeks. Intraperitoneal injections gave a mortality of about 15 per cent. The death rate among animals injected by the intramuscular or subcutaneous route was about 50 per cent. In contrast to the above extracranial injections those by the intracranial route resulted in a mortality of 100 per cent. By the extracranial methods it was noted that an injection

with a needle of large caliber and roughened surface more often resulted fatally than an injection with a small smooth needle; also, serial injections almost invariably killed, whereas single injections usually gave a low mortality.

It is evident that infection depends upon the laceration and entrance of the virus into the nerve trunks. In the nervous system the virus has a suitable medium in which to reproduce itself. Here it is not exposed to the rapid interchange of the body fluids; consequently, when it gains access to the nerve trunks it readily extends its invasion. The antagonistic action of the blood and lymph contributes a favorable influence in reducing the mortality from hydrophobia. We can readily assume that the bite of a rabid animal results in the implantation of a variable quantity of the virus. The virus must come in direct contact with the broken nerve fibers in order to reproduce itself and to extend along the nerve trunks, to the spinal cord and to the brain. As long as it is limited to the peripheral nerves it cannot reproduce itself in sufficient quantities to cause symptoms of the disease. Upon its entrance, however, to the spinal cord and brain there is presented an extensive trunk system through which it readily spreads, and in which the virus reproduces itself in such quantities as to cause characteristic symptoms, and, finally, a fatal termination.

The foregoing statements must not be construed as meaning that, even after a lapse of three days, cauterization is valueless. The virus which is not in direct continuity with the broken nerve trunk will be destroyed by the blood and lymph; moreover, owing to the slow progress of the virus, a reopening and thorough scrubbing of the wound, followed by cauterization with formalin or with fuming nitric acid—even after a lapse of three days—will be of great value in reducing the quantity of infecting virus.

The action of the body fluids on rabic virus was tested as follows: collodion sacs containing the standard suspension were placed in the peritoneal cavity of either rabbits or guinea-pigs. While bacterial organisms are sacked for the purpose of increasing their virulence, our results with the rabic virus on the contrary, were the exact opposite. It was found that when the virus was exposed to the peritoneal fluid its virulence was lost in from three

to six hours. The first sacking experiment consisted in introducing a sac into the peritoneal cavity of each of four rabbits. On each successive day following the operation one of the animals was anesthetized and the sac removed; 0.5 c.c. of its contents was injected intracranially into a guinea-pig. A portion of the original suspension used in this experiment was kept in the cold room, and injections from it served as control tests. On each successive day for four days a control animal was inoculated. All of the control animals died from rabies. On the other hand, none of those injected from the contents of the sacs succumbed to the disease. This experiment was verified by duplicate tests, and from these was proved the fact that the virus was destroyed in 24 hours. Moreover, subsequent experiments showed that the virus was no longer infectious when exposed to the body fluids for so short a time as four hours. In this set of experiments it was shown, by keeping the standard suspension at 37° C., that the body temperature alone does not destroy the infectivity of the virus for at least several days.

EXPERIMENT 1.

Exposure	Animal	Intracranial Inoculation	Death from Rabies
1 day	No. 1	0.5 c.c.	Lived
Control	" 2	" "	7th day
2 days	" 3	" "	Lived
Control	" 4	" "	6th day
3 days	" 5	" "	Lived
Control	" 6	" "	7th day
4 days	" 7	" "	Lived
Control	" 8	" "	8th day

This chart shows that exposure of the standard suspension to peritoneal fluid renders it non-virulent in one day. The control suspension was kept in the cold room.

EXPERIMENT 2.

Exposure	Animal	Intracranial Inoculation	Death from Rabies
2 hours	No. 1	0.5 c.c.	8th day
Control	" 2	" "	7th "
4 hours	" 3	" "	Lived
Control	" 4	" "	7th day
8 hours	" 5	" "	Lived
Control	" 6	" "	6th day
12 hours	" 7	" "	Lived
Control	" 8	" "	8th day

This chart shows that the virus, when exposed to the peritoneal fluid, is killed in at least four hours The control was kept at 37° C.

The following experiments show the effect of dialyzing a fresh standard suspension of rabic virus against running distilled water. These suspensions were dialyzed in collodion sacs prepared by the Novy method. In this series of experiments, as in all others, the only reliable method of showing the destruction of the virus is by the intracranial method of injection. Subcutaneous injection cannot be depended upon to show this destruction; as has already been pointed out, a virulent suspension is not always fatal when injected subcutaneously. At the termination of each series of exposure, an injection of the original untreated suspension served as a control.

EXPERIMENT 1.

Exposure	Guinea-Pig	Intracranial Injection	Death from Rabies
6 hours	No. 1	0.5 c.c.	6th day
12 "	" 2	" "	7th "
24 "	" 3	" "	6th "
Control	" 4	" "	" "

In this experiment a thick-walled collodion sac of 25 c.c. capacity was used.

EXPERIMENT 2.

Exposure	Guinea-Pig	Intracranial Injection	Death from Rabies
5 hours	No. 1	0.5 c.c.	7th day
10 "	" 2	" "	9th "
15 "	" 3	" "	7th "
24 "	" 4	" "	Lived
Control	" 5	" "	7th day

In Experiment 2 a sac of medium thickness with a capacity of 25 c.c. was employed.

EXPERIMENT 3.

Exposure	Guinea-Pig	Intracranial Injection	Death from Rabies
3 hours	No. 1	0.5 c.c.	8th day
6 "	" 2	" "	7th "
9 "	" 3	" "	8th "
12 "	" 4	" "	Lived
Control	" 5	" "	7th day

In Experiment 3 an extremely thin sac of 40 c.c. capacity was used.

EXPERIMENT 4.

Exposure	Guinea-Pig	Intracranial Injection	Death from Rabies
1 day	No. 1	0.5 c.c.	6th day
2 days	" 2	" "	7th "
3 "	" 3	" "	6th "
4 "	" 4	" "	8th "
Control	" 5	" "	" "

In Experiment 4 a three per cent suspension was dialyzed in a medium thick sac of 40 c.c. capacity.

EXPERIMENT 5.

Exposure	Guinea-Pig	Intracranial Injection	Death from Rabies
5 hours	No. 1	0.5 c.c.	7th day
10 "	" 2	" "	" "
15 "	" 3	" "	8th "
24 "	" 4	" "	7th "
Control	" 5	" "	" "

In Experiment 5 a very thin walled sac containing 40 c.c. of suspension was suspended in a cylinder containing an equal volume of distilled water.

EXPERIMENT 6.

Exposure	Guinea-Pig	Intracranial Injection	Death from Rabies
5 hours	No. 1	0.5 c.c.	8th day
10 "	" 2	" "	7th "
15 "	" 3	" "	8th "
24 "	" 4	" "	Lived
Control	" 5	" "	7th day

In Experiment 6 the same sac employed in Experiment 5 containing 40 c.c. of suspension was dialyzed against running distilled water.

It will be noted from these experiments that the thickness of the sac, the percentage of rabic brain in suspension, and the rate of flow of distilled water are important factors in effecting a destruction of the virus.

Our investigation of the protective value of the different methods of antirabic treatment consisted, first, in determining the M.L.D. of the fixed virus; second, in treating numerous sets of animals (rabbits and guinea-pigs) by the various methods, which are outlined in detail below; third, in subjecting these sets, after an interval of from two weeks to six months, to immunity tests.

In determining the M.L.D. of the fixed virus the following procedure of preparation is vital, inasmuch as it is essential to use from day to day fresh suspensions of uniform homogeneity. These suspensions were made from rabic brain obtained from stock rabbits immediately after death. The brain tissue was brought into suspension in the following manner: one gram of tissue and an equal volume of fine sharp sand were thoroughly crushed for five minutes with a heavy glass rod in a 150 c.c. test-glass. Then a few drops of physiological salt solution were added, and the mixture stirred for two minutes so as to bring the tissue into a thick suspension. After this two minutes of continual stirring, small quantities of salt solution were added, these additions alternating with mixing. Finally, larger quantities were added and stirred until there was a total of 100 c.c. The suspension, thus prepared, was kept in the cold room for 24 hours in order to free the supernatant liquid from lumps. About

75 c.c. of the supernatant suspension were then drawn off with a bulb pipette and filtered through a No. 595 Schleicher and Schüll filter paper. From this standard homogeneous filtrate (presumably 1–100) higher dilutions were made and injected. The procedure of preparing this standard suspension must be unvarying from day to day, so that a fixed quantity of a certain dilution of the suspension will represent the M.L.D.; should this dose vary from time to time no reliance can be placed on immunity tests, and such variations would indicate inaccuracy in preparing the basic standard 1–100 suspension.

Attempts were first made to determine the M.L.D. by the subcutaneous method of injection. From the standard 1–100 suspension, dilutions up to 1–500 of rabic brain were made which represented 1–200, etc. Of these suspensions one cubic centimeter was injected subcutaneously. Six sets of five guinea-pigs each were injected respectively with the standard suspension and its dilutions. In no two of these sets was the M.L.D. represented by the same dilution. In fact the 1–100 suspension was not always fatal; yet in the same set a higher dilution did result in death. Suspensions of 1–10, 1–20, etc., up to 1–100 also gave irregular results. It was, indeed, found that the 1–10, altho not always fatal itself, would, when diluted, produce death. A series of daily injections of dilutions up to and including 1–100 for three days invariably terminated in death. By this avenue of injection the incubation period was from 18 to 72 days. Intramuscular injections gave results very similar to the subcutaneous ones. By both of these methods of injections the M.L.D. could not be accurately determined; these, therefore, were not adopted in making immunity tests.

Realizing that the nerve tissue is the normal habitat of the virus, an attempt was now made to ascertain the M.L.D. by the intracranial method of injection. From a freshly prepared standard suspension, gradually decreasing doses were made by dilution; 0.5 c.c. of each dilution was injected intracranially. It was found that all injections of dilutions up to 1–20,000 were fatal; at times as high a dilution as 1–25,000 would kill. For the purpose of making immunity tests it was desirable to fix this M.L.D. within more narrow limits. At this point in the work it was found that the virus obtained from a rabbit which had died after several days of complete paralysis was not as virulent as that taken from one at the beginning of this stage. The rabic brain tissue at the beginning of the complete paralytic stage is not only more virulent, but its virulence is more uniform than are specimens taken after death. The animal may be killed at the beginning of this stage with ether, or by anesthesia and bleeding. The brain is then immediately removed and a standard 1–100 suspension is made as directed above. Suspensions of rabic brain made from rabbits killed in this stage were found—as the method of preparation was perfected—to be fatal in dilutions up to and almost invariably including 1–25,000 when injected intracranially in doses of 0.5 c.c. into rabbits and guinea-pigs. Numerous attempts to infect with a dilution of 1–26,000 failed; on the other hand an injection of 1–23,000 was occasionally the highest fatal dilution.

Having determined the M.L.D. of the fixed virus by the intracranial method of injection, the next step in demonstrating the protection afforded by the various methods of antirabic treatment was to immunize numerous sets of animals by these methods.

In making the immunity tests several sets of animals each of which had been immunized by one or another of the three methods were in some instances tested at the same time; the same dilutions, or subdilutions from the original 1–100 suspension were used in all sets tested. The purpose of such a procedure was to have the test conditions the same for the different sets. According to this method Tests 1 and 4 of the Pasteurian series were carried out at the same time as Tests 1, 3, and 4 of the Dialyzed series; Test 3 of the Pasteur and Test 4 of the Högyes; Test 3 of the Högyes and Test 21 of the Dialyzed; Test 1 of the Högyes and Tests 4 and 7 of the Dialyzed; Tests 2, 5, and 6 of the Pasteur and Tests 6, 12, 13, 15, and 19 of the Dialyzed series.

In the Högyes or the dilution method of treatment there were first prepared suspensions of 1–100 of fresh rabic brain in normal salt solution. In making these suspensions and their dilutions the procedure was that followed in working out the M.L.D.; there was, however, one point of difference: the animals were permitted to die. The dilutions and dosage on successive days are indicated as follows:

Day	Dilution	Dose c.c.	Day	Dilution	Dose c.c.
1st – 3d.....	1–10,000	4	18th.....	1–3,000	2
4th– 6th.....	1– 8,000	4	19th.....	1–2,000	2
7th– 9th.....	1– 6,000	4	20th.....	1–1,000	2
10th–13th.....	1– 5,000	2	21st.....	1– 500	2
14th–17th.....	1– 4,000	2	.....	.....	.....

The following tabulations show the immunity tests on four sets of animals treated with the above dilutions.

IMMUNITY TEST 1.

Animal	Dose	Death from Rabies
No. 1.....	1.65*M.L.D.	7th day
" 2.....	1.35 "	Lived
" 3.....	1.1 "	"
Control.....	1.0 "	9th day

Interval between treatment and test, 45 days.

IMMUNITY TEST 2.

Animal	Dose	Death from Rabies
No. 1.....	2.5 M.L.D	7th day
" 2.....	1.75 "	Lived
" 3.....	1.65 "	"
Control.....	1.0 "	6th day

Interval between treatment and test, 78 days.

* In all tabulations 1 M.L.D. represents 0.5 c.c. of a 1–25,000 dilution of rabic brain in physiological salt solution. 1 M.L.D. is equal to 0.02 milligram of brain tissue. 1.65 M.L.D. equals 0.5 c.c. of 1–15,000 dilution of brain tissue. The 0.5 c.c. of 1–15,000 dilution is equal to 0.033 milligram or 1.65 M.L.D.

IMMUNITY TEST 3.

Animal	Dose	Deatb from Rabies
No. 1	2.5 M.L.D.	9th day
" 2	1.85 "	Lived
" 3	1.65 "	"
Control	1.05 "	7tb day

Interval between treatment and test, 93 days.

IMMUNITY TEST 4.

Rabbit	Dose	Deatb from Rabies
No. 1	3.0 M.L.D.	10th day
" 2	2.2 "	" "
" 3	1.75 "	" "
" 4	1.4 "	Lived
" 5	1.25 "	"
Control	1.05 "	6th day
"	1.2 "	8tb day

Interval between treatment and test, 45 days.

SUMMARY OF ABOVE TABULATIONS.

Dose	Animals Tested	Deatb from Rabies	Protected
3.0 M.L.D.	1	1	
2.5 "	2	2	
2.2 "	1	1	
1.85 "	1		1
1.75 "	2	1	1
1.65 "	3	1	2
1.4 "	1		1
1.35 "	1		1
1.25 "	1		1
1.1 "	1		1

It will be noted in the summary of the above tabulations that the immunity conferred by this method of treatment will protect against about 1.5 M.L.D., and as will be shown, it is less than by the original method of Pasteur. Furthermore, an intracranial injection of any one of the dilutions used in the Högyes treatment will produce the disease in rabbits or guinea-pigs.

A modification of the above method, as used by some workers, consists in the use of freshly prepared virus—presumably 1–100—a method which is positively dangerous. The experience of thus inoculating the patient is demonstrated by Breggi. Attempts to produce immunity in rabbits and guinea-pigs in this laboratory with the 1–100 suspension resulted in infection from the treatment itself. In numerous sets of five animals so treated, infection resulted in two or more animals; at times, even all the animals in a given set died.

As carried out in this laboratory the Pasteur treatment consisted of injections of suspensions made from 14-day to 4-day desiccated rabic spinal cords. These suspensions were made in the following manner: From three-eighths to one-half of an inch of the required cord was transferred to a test-glass containing sharp sand, and thoroughly crushed with a glass rod. A few drops of normal salt solution were then added, and a thick suspension was made by continuing the crushing process. At

intervals more salt solution was added, the additions alternating with mixing until a total of 30 c.c. was added. By following this procedure a thoroughly homogeneous suspension is obtained. The spinal cord suspensions and the dosage were as follows:

Day	Spinal Cord	Dose c.c.	Day	Spinal Cord	Dose c.c.
1st	14–13	6	12th	6	2
2d	12–11	6	13th	5	2
3d	10–9	6	14th	4	2
4th	8	2	15th	6	2
5th	7	2	16th	5	2
6th	6	2	17th	4	2
7th	5	2	18th	6	2
8th	4	2	19th	5	2
9th	6	2	20th	4	2
10th	5	2	21st	4	2
11th	4	2			

The following tabulations show the immunity tests on six sets of animals treated by the Pasteur method:

TEST 1.

INTERVAL: 43 DAYS.

Dose	Guinea-Pig	Death from Rabies
3.0 M.L.D	No. 1	7th day
2.5 "	" 2	Lived
2.05 "	" 3	7th day
1.65 "	" 4	Lived
1.5 "	" 5	"
1.05 "	Control	7th day

TEST 2.

INTERVAL: 51 DAYS.

Rabbit	Death from Rabies
No. 1	8th day
" 2	52d "
Control	6th "

TEST 3.

INTERVAL: 60 DAYS.

Dose	Rabbit	Death from Rabies
3.0 M.L.D		
2.5 "	No. 1	8th day
2.05 "	" 2	" "
1.75 "	" 3	Lived
1.4 "	" 4	"
1.0 "	Control	7th day

TEST 4.

INTERVAL: 68 DAYS.

Guinea-Pig	Death from Rabies
No. 1	8th day
" 2	" "
" 3	Lived
" 4	"
Control	8th day

TEST 5.

INTERVAL: 101 DAYS.

Dose	Guinea-Pig	Death from Rabies
6.0 M.L.D		
3.0 "	No. 1	11th day
2.5 "	" 2	10th "
1.5 "	" 3	Lived
1.25 "	Control	9th day
1.05 "	"	" "

TEST 6.

INTERVAL: 180 DAYS.

Guinea-Pig	Death from Rabies
No. 1	8th day
" 2	Lived
" 3	9th day
" 4	Lived
Control	8th day

SUMMARY OF ABOVE TABULATIONS.

Dose	Animals Tested	Death from Rabies	Protected
6.0 M.L.D.	1	1	
3.0 "	4	4	
2.5 "	3	2	1
2.05 "	6	5	1
1.75 "	2		2
1.65 "	2	1	1
1.5 "	3		3
1.4 "	1		1

These tabulated experiments are the last 6 of a series of 17 which were carried out in our investigation of the protection afforded by the Pasteur treatment. The last 6 are presented owing to the fact that in these the results are more reliable than in the earlier numbers, inasmuch as the technic of testing was perfected as the series progressed.

In the summary it will be observed that the Pasteur treatment protects against about 2 M.L.D. of the fixed virus injected by the intracranial method. The immunity produced by this method of treatment may be expected to protect against an amount of street virus subcutaneously introduced equivalent to 2 M.L.D. of fixed virus injected intracranially, or a smaller amount which, during an interval of exposure, has increased to an equivalent of 2 M.L.D. We may then say that a case is hopeless if the street virus introduced into the wound is equal to 3 M.L.D., or if a small dose of the original virus multiplies to 3 M.L.D. during an interval of exposure.

The vital preliminary to the vaccine treatment is the proper cauterization of the wound; a second, and equally important step, is immediate antirabic treatment. If in a severe lacerating bite an equivalent of 3 M.L.D. of the fixed virus, injected intracranially, is introduced into the wound, one-half or even more of the infecting virus may be destroyed by proper cauterization, and the subsequent preventive injections will protect against that remaining. The value of cauterization with formalin, especially in face bites and lacerating ones on other parts of the body, cannot be overestimated. This, followed by the vaccine treatment, should reduce the mortality from hydrophobia to zero.

In addition to the work on the Högyes and the Pasteur methods of antirabic treatment we present the experimental and practical

results of a method which developed from our original research with rabic virus in this laboratory. For the purpose of designation this will be termed the method of dialysis. The destructive action effected by dialyzing the virus against running distilled water has already been explained. The preparation of the vaccine by this method is based on the use of a thoroughly homogeneous suspension of rabic brain and the employment of standardized collodion sacs. The technic of preparing the homogeneous suspension is the same as that given in detail under the discussion of the M.L.D.; there are, however, two points of exception: (1) in place of physiological salt solution, distilled water is used; (2) the animal is not killed at the beginning of the complete paralytic stage, but is permitted to die from the disease.

In the standardization of collodion sacs the procedure is as follows: Thin sacs are prepared by the Novy method. They are sterilized in the autoclav at 105° C. for 20 minutes. These are then filled to the glass line with the standard suspension which is dialyzed against running distilled water. At intervals of 8, 16, and 24 hours a few cubic centimeters are withdrawn from each sac, and 0.5 c.c. of each specimen is injected intracranially into a guinea-pig. After the injection from the 24-hour exposure has been made the contents of the sacs are withdrawn. They are then filled with 0.4 per cent formaldehyd solution, and set aside for future use awaiting the results of the animal inoculations. At the termination of 10 days the results of these inoculations indicate the rate of dialysis in the various sacs. If in any case the injection from the 24-hour exposure caused death from rabies in the test animal, the sac in which this suspension had been dialyzed was discarded. On the other hand, those sacs in which the virus was destroyed within 24 hours were retained and made use of in preparing further quantities of vaccine.

A similar method which does not require such a prolonged dialysis is that of first destroying the active virulent virus by the use of any of the specific disinfectants. For this purpose the standard homogeneous suspension in distilled water is treated with 0.1 per cent formaldehyd. After sedimentation for three hours the supernatant liquid is drawn off and dialyzed until the test for formaldehyd becomes negative.

The vaccine obtained by either of these methods of dialysis is tested as follows: first, by intracranial injections into guinea-pigs, for both infectivity of the virus and bacterial contamination; second, on artificial media for bacterial contamination. As a preservative 0.1 per cent tricresol is added.

Of the following 25 immunity tests those animals in Tests 1, 2, 11, 17, 18, and 25 were immunized with vaccine which was first treated with formaldehyd and then dialyzed, whereas those in the remaining Tests (3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 19, 20,

21, 22, 23, and 24) were immunized with vaccine prepared by dialysis alone.

The accompanying tabulations are arranged according to the immunizing dose, the number of days treated, and the interval elapsing between the treatment and the immunity tests. The sets of animals receiving small doses for a brief time and having a short interval appear first in the series.

TEST 1.

Dose	Guinea-Pig*	Death from Rabies
3.0 M.L.D.	No. 1	Lived
2.5 "	" 2	"
2.05 "	" 3	"
1.75 "		
1.60 "	" 4	"
1.30 "	" 5	"
1.20 "		
1.05 "	Control	7th day
1.0 "		

TEST 2.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
" 4	"
" 5	"
Control	8th day
"	7th "
"	10th "

* Protective treatment: 2 c.c. dialyzed vaccine on 11 successive days. Interval: 43 days.
† Protective treatment: 2 c.c. dialyzed vaccine on 11 successive days. Interval: 105 days.

TEST 3.

Dose	Guinea-Pig*	Death from Rabies
4.0 M.L.D.	No. 1	Lived
3.0 "	" 2	8th day
2.5 "	" 3	Lived
2.05 "	" 4	"
1.75 "	" 5	"
1.65 "		
1.4 "	" 6	"
1.0 "	Control	7th day

TEST 4.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
" 4	"
Control	7th day

* Protective treatment: 2 c.c. of dialyzed vaccine (which had been kept in the cold room for five months) on 12 successive days. Interval: 57 days.
† Protective treatment: 2 c.c. of dialyzed vaccine (which had been kept in the cold room for three months) on 14 successive days. Interval: 48 days.

TEST 5.

Dose	Guinea-Pig*	Death from Rabies
6.0 M.L.D.		
4.0 "		
3.0 "		
2.5 "	No. 1	Lived
1.65 "	" 2	"
1.35 "	" 3	"
1.05 "		
1.0 "	Control	8th day

TEST 6.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
Control	9th day

* Protective treatment: 3 c.c. dialyzed vaccine (1 part rabic brain to 25 parts water) on 9 successive days. Interval: 37 days.
† Same animals as in Test 5, tested again after an interval of 120 days.

TEST 7.

Dose	Guinea-Pig*	Death from Rabies
4.0 M.L.D.		
3.0 "		
2.5 "	No. 1	Lived
1.6 "	" 2	"
1.05 "		
1.0 "	Control	8th day

TEST 8.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
Control	9th day

* Protective treatment: 3 c.c. dialyzed vaccine on 10 successive days. Interval: 37 days.

† Test 7 animals tested again after an interval of 120 days.

TEST 9.

Dose	Guinea-Pig*	Death from Rabies
3.0 M.L.D.	No. 1	Lived
2.5 "	" 2	2d day‡
2.05 "		
1.65 "	" 3	Lived
1.35 "	" 4	"
1.0 "	Control	7th day

TEST 10.

Guinea-Pig†	Death from Rabies
No. 1	10th day
" 2	Lived
" 3	"
Control	7th day

* Protective treatment: 3 c.c. dialyzed vaccine (which had been kept in the cold room for 3 months) on 14 successive days. Interval: 58 days.

† These are the remaining 3 animals from Test 9 tested again 25 days later.

‡ Cultures from heart's blood showed a bacillus.

TEST 11.

Dose	Guinea-Pig*	Death from Rabies
6.0 M.L.D.		
4.0 "	No. 1	Lived
3.0 "	" 2	9th day
2.5 "	" 3	Lived
2.0 "	" 4	"
1.75 "	" 5	"
1.5 "	" 6	"
1.0 "	Control	8th day

TEST 12.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
Control	8th day

* Protective treatment: 3 c.c. dialyzed vaccine (which had been kept in the cold room for 3 months), on 14 successive days. Interval: 72 days.

† Protective treatment: 4 c.c. dialyzed vaccine, intraperitoneally, on 16 successive days. Interval: 120 days.

TEST 13.

Dose	Guinea-Pig*	Death from Rabies
3.5 M.L.D.		
3.0 "	No. 1	Lived
2.5 "		
2.05 "	" 2	"
1.5 "	" 3	"
1.02 "	Control	7th day

TEST 14.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
Control	7th day

* Protective treatment: 5 c.c. dialyzed vaccine, injected intraperitoneally, on 7 successive days. Interval: 60 days.

† Animals in Test 13 re-tested 40 days later.

TEST 15.

Dose	Rabbit*	Death from Rabies
5.0 M.L.D.		
3.5 "		
3.0 "	No. 1	Lived
2.8 "		
2.05 "	" 2	"
1.5 "	" 3	"
1.05 "	Control	7th day

TEST 16.

Rabbit†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
Control	7th day

* Protective treatment: 5 c.c. dialyzed vaccine on 7 successive days. Interval: 20 days.

† Animals in Test 15 re-tested 40 days later.

TEST 17.

Dose	Guinea-Pig*	Death from Rabies
3.0 M.L.D.	No. 1	62nd day
2.5 "	" 2	Lived
1.95 "	" 3	"
1.65 "	" 4	"
1.35 "	" 5	"
1.0 "	Control	7th day

TEST 18.

Guinea-Pig†	Death from Rabies
No. 1	7th day
" 2	Lived
" 3	"
" 4	"
" 5	8th day
Control	7th "

* Protective treatment: 5 c.c. dialyzed vaccine subcutaneously on 8 successive days. Interval: 20 days.

† Protective treatment: same as Test 17 except that the injections were made intraperitoneally. Interval: 20 days.

TEST 19.

Dose	Guinea-Pig*	Death from Rabies
8.0 M.L.D.		
6.0 "	No. 1	Lived
3.5 "	" 2	"
2.05 "	" 3	"
1.0 "	Control	8th day

TEST 20.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
Control	7th day

* Protective treatment: 5 c.c. dialyzed vaccine intraperitoneally on 8 successive days. Interval: 90 days.

† Animals in Test 19 re-tested 40 days later.

TEST 21.

Dose	Guinea-Pig*	Death from Rabies
3.0 M.L.D.		
2.5 "	No. 1	Lived
2.05 "	" 2	"
1.75 "	" 3	"
1.05 "	Control	8th day
1.0 "		

TEST 22.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
Control	8th day

* Protective treatment: 5 c.c. dialyzed vaccine (which had been kept in the cold room for 10 weeks) intraperitoneally on 9 successive days. Interval: 70 days.

† Animals in Test 21 re-tested 150 days later.

TEST 23.

Dose	Guinea-Pig*	Death from Rabies
3.0 M.L.D.	No. 1	Lived
2.0 "	" 2	"
1.35 "	" 3	"
1.0 "	Control	7th day

TEST 24.

Guinea-Pig†	Death from Rabies
No. 1	Lived
" 2	"
" 3	"
Control	7th day

* Protective treatment: 5 c.c. dialyzed vaccine intraperitoneally on 15 successive days. Interval: 44 days.

† Protective treatment: same as Test 23 except that the injections were made subcutaneously. Interval: 44 days.

TEST 25.

Dose	Guinea-Pig*	Death from Rabies
6.0 M.L.D.	No. 1	Lived
3.0 "	" 2	"
2.05 "	" 3	"
1.05 "	Control	8th day

* Protective treatment: 5 c.c. dialyzed vaccine intraperitoneally on 16 successive days. Interval 120 days.

SUMMARY OF THE ABOVE TABULATIONS.

Dose	Animals Tested	Death from Rabies	Protected
8 M.L.D.	1		1
6 "	5		5
5 "	1		1
4 "	8		8
3.5 "	1		1
3 "	19	5*	14
2.8 "	1		1
2.5 "	15		15
2.05 "	9		9
2 "	3		3
1.95 "	2		2
1.75 "	4		4
1.65 "	4		4
1.60 "	4		4
1.5 "	3		3
1.4 "	2		2
1.35 "	6	1†	5
1.3 "	2		2

* These deaths may be partially explained by reason of the fact that two of the series were treated with old vaccine (2 and 5 months old), and in two others the interval between treatment and immunity tests was comparatively short.

† Death of this animal is apparently an example of no response to treatment.

It will be noted in the summary of the above tests that this method of treatment will protect against as much as 8 M.L.D. Furthermore, the vaccine so prepared, when injected intracranially, will not produce the disease; therefore, subcutaneous injections of the dialyzed vaccine are not only perfectly safe as a prophylactic

measure, but, as has been shown, they confer the highest degree of immunity. It is possible to reproduce rabies in animals by intracranial injection of a suspension made from a six-day desiccated cord, and an intracranial injection of any one of the dilutions used in the Högyes treatment will result in death from rabies. By this new method, however, of preparing the vaccine, it is impossible to produce the disease in animals even by intracranial injections, thus absolutely eliminating all danger of infection by subcutaneous injections, while at the same time, this method confers the highest degree of immunity that can be obtained with safety. A series of 12 successive daily injections of the standard dialyzed vaccine gives the same degree of immunity as is obtained by the intensive form of the Pasteur treatment, which consists of 21 daily injections. The vaccine prepared by the method of dialysis is of especial value, not only in the case of severe bites, particularly those on the face, but also in those cases which do not report for treatment earlier than two weeks after having been bitten.

In conclusion it may be stated that, in this Institute and elsewhere in the United States, over 800 persons bitten by animals suspected of having rabies were treated by this new method. Of this number 62 per cent were bitten by animals proved by laboratory examination to have been rabid. In this series of patients the treatment has been efficient in protecting against hydrophobia in every case. The treatment consisted of two cubic centimeters daily for from 15 to 25 days. A local anaphylactic reaction at the point of injection is not uncommon among patients treated by the dialyzed vaccine. It manifests itself on the seventh or eighth day by redness, slight swelling, and itching. In no case is the reaction marked, and its duration is about 24 hours. This phenomenon is not due to rabic virus, or its products of cleavage, but is common to a series of injections of any foreign proteins—in this case brain cell protein. In cases of trivial scratches and those in which there was a possibility of the saliva having entered an open wound a 15-day course of treatment was considered sufficient. In the average case from 18 to 21 daily treatments were

prescribed; whereas in cases of severe uncauterized bites on uncovered surfaces of the body, especially on the face, and in which there had been a delay of two weeks or more in applying for treatment it was considered desirable to give two treatments daily for the first four days, and these were followed by single daily injections through the twenty-fifth day.

I wish to thank Professor Victor C. Vaughan and Professor Frederick G. Novy for their suggestions and invaluable criticism on this work, and to express my appreciation of the conscientious assistance of Dr. Robert Stark, Mr. Quinter O. Gilbert, and Mr. C. G. Sinclair.

TUMORS OF GROUND SQUIRRELS (CITELLUS BEECHEYI).*

GEORGE W. MCCOY.

(From the United States Public Health Service, Washington, D.C.)

In the course of the examination of ground squirrels at the Federal Plague Laboratory at San Francisco, California, for the purpose of detecting plague infection, several tumors have been observed which will be described briefly in this paper.

New growths evidently occur much less frequently in these rodents than in rats, the experience[1] being that a neoplasm is found about once in each 1,000 rats, while only eight tumors were found among 250,000 ground squirrels, or but one in over 30,000 animals. A few other growths were found, the gross appearances of which led us to regard them as tumors but which the microscopical examination relegated to the group of granulomata.

Sections of the growths were submitted to Professor Wm. J. Ophuls, of the Department of Pathology of Leland Stanford Junior University, who kindly gave me the benefit of his large experience in tumor work and furnished me with notes on which the histological diagnoses are based chiefly.

TUMORS OF THE SUBCUTANEOUS TISSUE.

Lipoma.—Grown; female. A well-defined, flattened, oval, fatty mass just under the skin on the left side midway between the axilla and the groin. Dimensions, 0.5×1×2 cm.

The sections showed nothing beyond the loose connective tissue stroma which formed the supporting structure of fatty tissue.

Fibro-sarcoma.—Grown; female. A firm, grayish-white growth in the subcutaneous tissue of the inguinal region. Dimensions, 2×3×4 cm.

The microscopical examination showed a fibrous stroma with an abundance of round cells. The cells are fairly uniform in size

*Received for publication September 14, 1913.

[1] McCoy, *Jour. Med. Research*, 1909, 21, p. 285; Woolley and Wherry, *ibid.*, 1911, 25, p. 205.

and shape. The border of the growth merges into the connective tissue of the part.

TUMOR OF MEDIASTINUM.

Sarcoma.—Grown; female. A soft, almost diffluent, pinkish-white mass, which probably springs from the thymus, occupies the whole anterior mediastinum.

The growth is made up of round nuclei with merely a suggestion of connective tissue formation in some parts and in others a well-defined but loose, irregular, fibrous tissue network. Well-defined blood vessels are lacking. There is no trace of Hassel's corpuscles tho they are present in abundance in control tissue (thymus) from normal squirrels.

TUMORS OF LIVER.

Adenoma.—Grown; female. The liver presents two firm, lobulated, buff-colored masses each about $0.5 \times 1 \times 2$ cm., and another mass of the same nature but only about the size of a pea. The growths project abruptly from the general contour of the liver.

The growth is made up of epithelial cells like those of the liver, except that they are larger, irregular in shape and size, and lack the regular arrangement of liver cells. In portions the growth appears to be marked off from the liver structure by a band of connective tissue rich in cells.

Adenoma.[1]—Grown; female. The lower surface of the liver presents a reddish growth about 4 cm. in diameter.

This growth is much more atypical than the preceding one. The cells are larger and more irregular than in the first case. Large blood spaces are present, some of which lack well-defined walls. Portions of the growth show degenerative change, probably fatty in nature.

TUMOR OF KIDNEY.

Sarcoma (*angio-sarcoma?*).—Grown; female. Springing from the inner and front surface of the right kidney is a soft, whitish mass about 2.5×3.5 cm. The growth shows reddish streaks. The kidney tissue presents no gross evidence of disease. The

[1] The diagnosis of *angioma* might be justified almost equally well in this case.

growth is made up of a delicate framework filled with round or oval nuclei. In places large capillary vessels lie in the center of cellular masses.

TUMORS OF FEMALE GENITALIA.

Sarcoma (alveolar).—Grown; female. A grayish-white, lobulated growth apparently springing from the right ovary or from the extremity of the right division of the uterus. The growth is tough with a tendency to softening in the center.

Microscopically this growth presents a fibrous, somewhat cellular framework with nests of mononuclear cells.

Sarcoma (alveolar).—Grown; female. A grayish-white, lobulated mass at the distal end of the right horn of the uterus. The growth is firm, fleshy, and cuts readily. Dimensions, 1×2×4 cm.

This growth resembles the preceding one. The framework is less cellular. The structure is very vascular.

SUMMARY.

Eight neoplasms were found in 250,000 ground squirrels (*Citellus beecheyi*).

The tumors were all found in females.

The microscopic diagnoses and the location of the growths were as follows:

1 lipoma in the subcutaneous tissue;
1 fibro-sarcoma in the subcutaneous tissue;
1 sarcoma of mediastinum;
1 sarcoma of kidney;
2 sarcomas of female genitalia (internal);
2 adenomas of liver.

OBSERVATIONS ON ANTIBODY FORMATION IN TYPHOID.*

VIRGIL H. MOON.

(From the Memorial Institute for Infectious Diseases, Chicago.)

It has been shown repeatedly that there is a marked increase of antibodies in the blood following inoculation against typhoid fever. In a previous article[1] I have described the course of the curve of agglutination for a period of two years following such inoculation. At the end of that time the agglutinins have decreased to practically the normal amount which was present before the injections of killed bacilli were made. If we assume that resistance to disease runs parallel with the amount of antibodies present in the serum we would conclude that the artificially induced resistance to typhoid had practically disappeared after two years. However such an assumption is not justifiable. The rapidity with which antibodies are produced is as important a factor in the production of resistance as is the relative amount of antibodies present. Cole[2] observed that a guinea-pig which had been previously injected with typhoid bacilli on being reinjected responded promptly by a rapid and abundant formation of antibodies, while a normal guinea-pig treated similarly responded slowly and gave a comparatively low curve. Other observers have recorded similar results. The following experiment was made in order to observe the behavior of the agglutinin curve in human subjects.

Persons who had previously received antityphoid inoculation or had had typhoid fever were given a single injection of killed typhoid bacilli. Other persons of about the same age who had received no previous inoculation and had not had typhoid fever were injected similarly. The blood was tested daily for agglutinins by determining at what dilution the serum would produce noticeable agglutination in a suspension of young, motile, typhoid bacilli. This was determined by microscopic examination after 45 minutes at 37° C.

* Received for publication August 6, 1913.

[1] *Jour. Amer. Med. Assn.*, 1913, 60, p. 1764.

[2] *Ztschr. f. Hyg. u. Infectionskrankh.*, 1904, 66, p. 367.

The results are represented graphically in the following charts which trace the curve of agglutination for 15 days following the injection. The vertical lines mark days while the horizontal lines indicate the dilution of the sera.

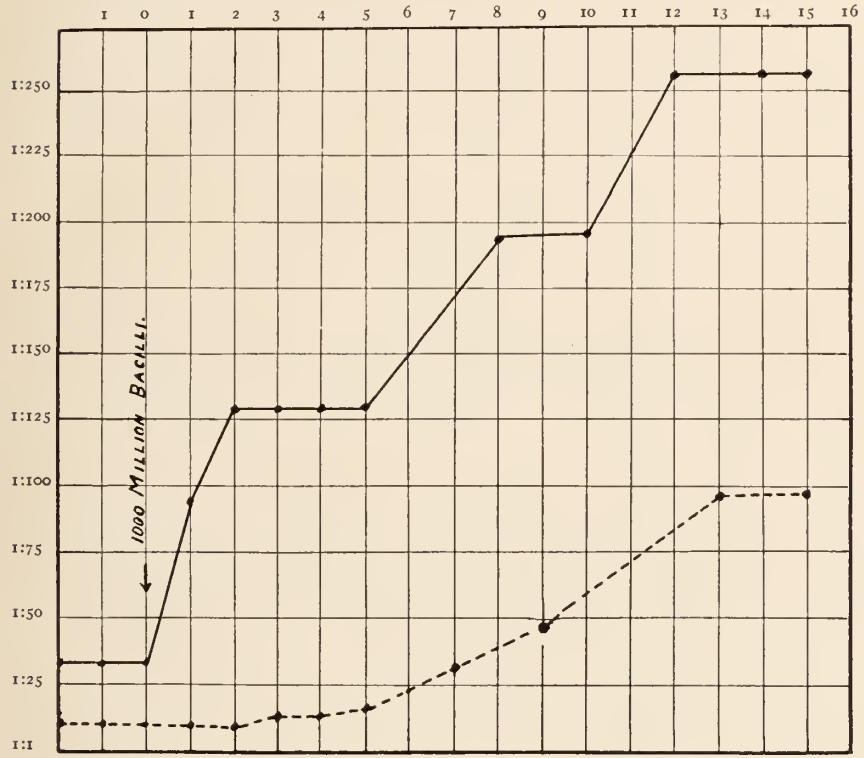

CHART 1.—The solid line represents the curve of a person who had received typhoid inoculation two and one-half years previously. The line of dashes represents the control. Both received an injection of 1,000 million killed bacilli on the day indicated.

As shown by these curves there is a distinct difference in the rapidity with which human beings who have previously been immunized form antibodies as compared with those who have not. It would seem as if the previously immunized person remains more sensitive to the antigenic influence of typhoid bacilli and responds more quickly by the production of antibodies when the bacilli are introduced into the system. Such a result is in keeping with our knowledge of the phenomena of allergy. That this condition would

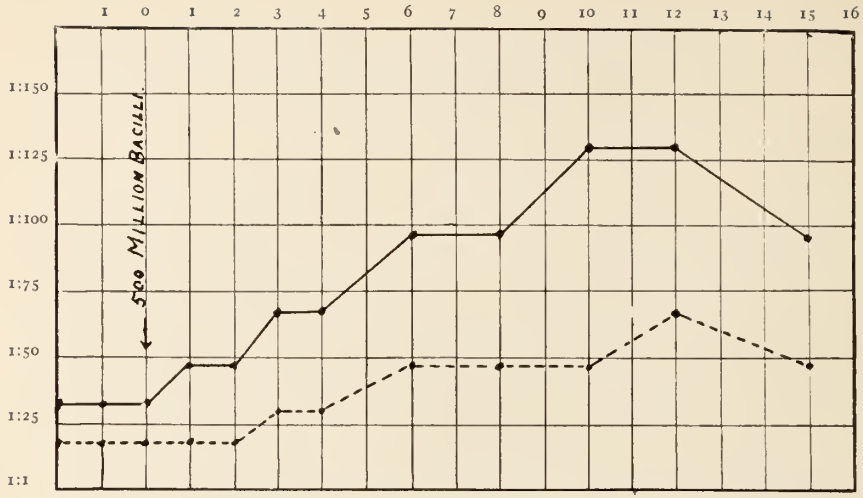

CHART 2.—The solid line represents the curve of a person who had received protective inoculation one and one-half years previously. The line of dashes represents the control. 500 million killed bacilli were injected in each on the day indicated.

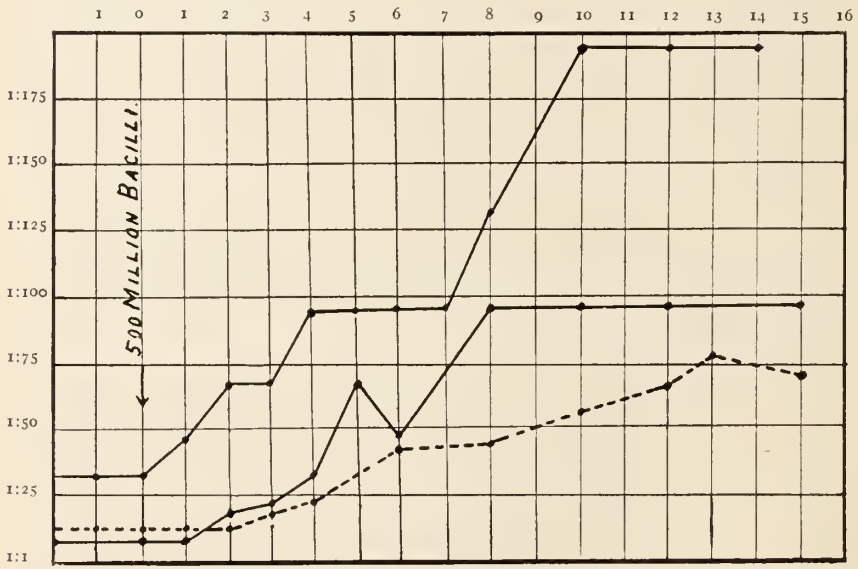

CHART 3.—The solid lines represent the curves of two persons who had had typhoid fever 12 years previously. The line of dashes represents the average curve of two controls who had no typhoid history. 500 million killed bacilli were injected on the day indicated.

contribute to the resistance against typhoid and the readiness with which the body would overcome the bacilli is easily understood. Chart 3 would indicate that the same condition exists after an attack of typhoid fever, but that the degree of excitability is slight after a period of 12 years.

Rolly and Meltzer[1] found that typhoid agglutinins and bacteriolysins are produced more abundantly in rabbits under conditions of experimental hyperthermia. On the other hand, Graziani[2]

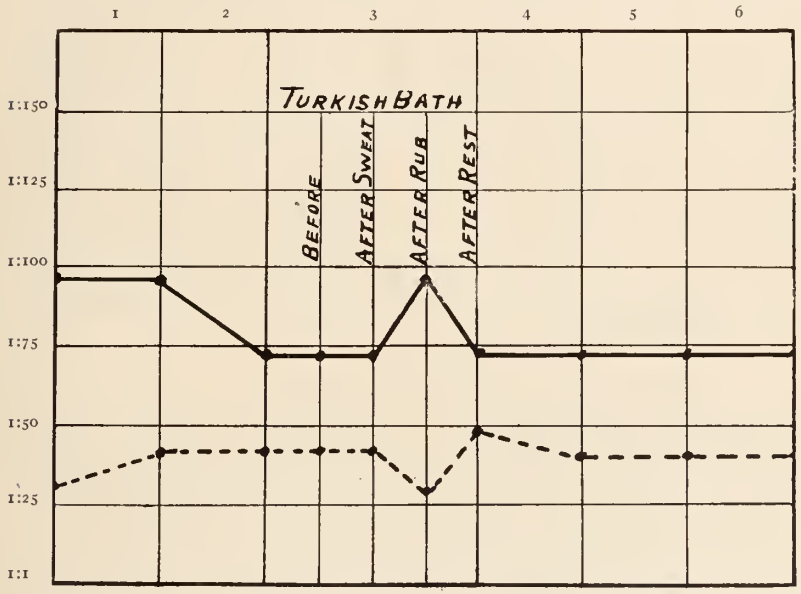

CHART 4.—The two curves here shown represent the agglutinins in the sera of two persons before, during, and after the Turkish bath.

found that when rabbits are injected with filtrates of typhoid cultures and are subsequently kept at different temperatures, those kept at a temperature slightly above freezing developed more agglutinins than those kept at higher temperatures.

Von Leube[3] found that patients recovering from typhoid fever showed a material increase in the agglutinin content of the blood after hot baths, 40° C. for 30 minutes.

[1] *Deutsch. Arch. f. kl. Med.*, 1909, 94, p. 385.

[2] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1907, 42, p. 633.

[3] *Verhandl. d. deutsch. Kongresses f. inn. Med.*, 1910, 27, p. 218.

These observations suggested the question whether a transient elevation of temperature such as may be attained in a Turkish bath, would cause any perceptible change in the antibody content of the sera of persons immunized to typhoid bacilli. Accordingly persons who had received antityphoid inoculation several weeks previously were selected for experiment. They were taken through the routine of the Turkish bath which consisted of 30 minutes in the dry room at a temperature of 180° F., 20 minutes in the steam room at a temperature of 130° F., followed by massage, rub, shower bath, and an hour's rest. Samples of serum taken before, during, and after the Turkish bath were tested for agglutinins as in the experiments previously described. Chart 4 represents the curves of two persons so treated. The only variation observed occurred in the samples taken after the massage and rub, and these variations were of opposite character in the two cases. This variation was transient and so slight in degree that it might be accounted for as due to the greater concentration of the serum following profuse perspiration, or to experimental error.

SUMMARY.

We should conclude then that the protective effect of antityphoid inoculation lasts for a longer period than two years, and that this effect is due not so much to the persistence of antibodies in the blood as to the promptness with which new antibodies are formed in the presence of typhoid proteins.

Turkish baths probably do not materially affect the concentration of agglutinins in the sera of persons inoculated with typhoid bacilli.

THE ETIOLOGY OF ACUTE RHEUMATISM, ARTICULAR AND MUSCULAR.*

E. C. Rosenow.

(From The Memorial Institute for Infectious Diseases, Chicago.)

The view that rheumatism is due to a specific streptococcus as claimed by Poynton and Paine[1] and others, has not been generally accepted because many investigators (Phillip,[2] Cole,[3] Beattie,[4] and others) failed entirely to obtain streptococci from the joints while others as Loeb[5] only rarely succeeded. No one has isolated the organism in a considerable number of consecutive, uncomplicated cases of rheumatism during life.

I now wish to report the results of cultures from the blood and the lesions in cases of articular and of muscular rheumatism, the chief characteristics of the organisms isolated, and results of experiments.[6]

Technic of Cultures.

The skin was sterilized with tincture of iodin, and the joints aspirated with a glass syringe through a needle of small caliber. The pain was slight and the patients were afforded relief from pain whenever the amount of fluid obtained was considerable. The fluid was emptied into sterile test tubes through the flamed end of the syringe and not through the needle. The mouth of the test tubes was thoroughly flamed and kept open as short a time as possible. The material was then taken to the laboratory and cultures made as soon as possible. In a number of cases cultures were made immediately at the bedside, but the number of organisms isolated was no greater than in those made later in the laboratory. At first the cultures were made both under aerobic and anaerobic conditions in plain broth, ascites broth, ascites-dextrose broth, blood broth, litmus milk, ascites fluid (heated and unheated), Löffler's blood serum slants, blood agar plates and slants.

The fact that the lesions in rheumatism occur in relatively avascular regions suggested that the infecting organism might be sensitive to oxygen pressure. In order to meet this possibility the joint fluid was inoculated into tubes containing tall columns of ascites-dextrose agar, the top of which would give aerobic conditions, while the bottom would be anaerobic on account of the oxygen consuming property of the leukocytes

* Received for publication September 10, 1913.

[1] *Lancet*, 1900, 2, p. 861; 1910, 1, pp. 152 and 1528.

[2] *Deutsch. Arch. f. klin. Med.*, 1903, 76, p. 150.

[3] *Jour. Infect. Dis.*, 1904, 1, p. 714.

[4] *Brit. Med. Jour.*, 1906, 2, p. 1781.

[5] *Arch. Int. Med.*, 1908, 2, p. 266.

[6] A preliminary report was published in *Jour. Am. Med. Assn.*, 1913, 60, p. 1229.

in the exudate, the space between representing a gradual transition from one condition to the other. It was soon found that this method gave by far the best results. The standard 2 per cent agar, 0.4 to 0.6 per cent acid to phenolphthalein, and containing 0.2 to 1 per cent dextrose, was used, 7 to 8 c.c. being placed in each tube. The agar was melted and boiled for a time to drive off oxygen, cooled to 50° C., and 2 to 3 c.c. heated ascites fluid (60° C. 24 hours) added to each. After being cooled to 40° C., from 0.1 to 1 c.c. of the joint fluid was inoculated, depending on the amount at hand, and mixed with the agar. The tube was plunged into cold water to "set" and then placed at 37° C. Growth usually was present at the end of 48 hours, but sometimes it would not take place until after three or more days. The cultures (aerobic and anaerobic) made in liquid and on the surface of solid media yielded positive results in only an occasional case, while the method just described yielded positive results in the case of one or more joints in 14 of 16 cases. That the oxygen requirement is the chief factor to explain this difference in my results and the negative results of others is indicated also by the fact that the colonies never developed above 0.5 cm. from the top and never below 2 cm. from the bottom of the agar tubes. The largest number of colonies developed between 1.5 cm. from the top and 3.5 cm. from the bottom.

THE RESULTS.

An account of the cultures of the joint fluid from one case will illustrate the results. There were aspirated 18 c.c. of fluid which was inoculated into plain broth, dextrose broth (with and without ascites fluid), on the surface of blood agar slants (aerobic and anaerobic), and into ascites-dextrose agar, four tubes of which received one-third of the fluid. All liquid cultures remained sterile, one blood agar slant (anaerobic) yielded a positive result, while in the ascites-dextrose agar on the other hand there developed an average of nine colonies per cubic centimeter of fluid. No colonies developed above one centimeter from the top of the agar or below three centimeters from the bottom.

The joint fluids obtained were only moderately turbid, due to the presence of leukocytes and a few endothelial cells. Smears showed unmistakable diplococci in only three cases. The two cases in which cultures gave negative results were fever free and convalescing at the time the cultures were made. All the cases were typical examples of acute rheumatic fever and were not selected except as to time.[1]

It was early learned that cultures made soon after a joint

[1] The cases studied occurred chiefly in the Cook County and Presbyterian Hospitals, and I wish here to express my appreciation to the medical staffs for the privileges accorded me, and to Dr. Falls and Dr. Phemister for aid in obtaining material for cultures.

became involved or soon after an exacerbation showed more organisms than at other times. The number of organisms obtained from the fluid, however, was never more than 15 per cubic centimeter.

The results in the following case are interesting. Carpenter, 36 years old, began to have severe pain in groins and right hip after severe exposure four days before. Left knee became very painful and swollen the following day, and the right knee two days later. The left wrist became involved the following night. Free fluid was easily demonstrable in the joints involved and a red, very tender, swollen area was found over the lower end of the ulna. Both knees and wrist joints were aspirated. A needle was inserted in various directions into the red area over the ulna and by means of strong suction a small quantity of bloody fluid was obtained. This was inoculated in ascites-dextrose agar at once. Cultures from the joint fluid were made in the same way. The fluid from the right knee yielded two colonies per 20 c.c., from the left knee two colonies per 12 c.c., from the wrist three colonies per 2 c.c., while the small amount (approximately 0.05 c.c.) of bloody fluid from the red area yielded 10 colonies.

In the cultures from a number of joints there developed a few colonies of a large gram-positive aerobic bacillus which resembled *B. subtilis*. This was probably due to air contamination.

Blood cultures were made in seven cases. In all fever was present, ranging from 99.5° to 102° F. when the cultures were made. In only one was endocarditis present. Three of the cultures were made in the usual way, the blood being inoculated at once into plain broth, ascites-dextrose broth and into plain and ascites-dextrose agar. Two of these cultures were sterile while the third yielded two very small colonies in the fibrin clot in the dextrose broth cultures. In the other four cases the cultures were made by drawing 30 c.c. of blood into 15 c.c. of 2 per cent sodium citrate in NaCl solution. In order to get rid of the hemoglobin the red blood corpuscles were now hemolyzed by adding approximately 100 c.c. of distilled water. This fluid was now centrifugated at high speed and the sediment inoculated at once in broth and ascites-dextrose agar. From two to 12 colonies developed by this method in three of the cases. The cultures in the broth proved sterile in all four. Simultaneous blood and joint cultures were made in four cases. The joint cultures were positive in all of these but blood cultures were sterile in two. The organism was isolated from the tonsil at the height of the attack in one case.

While the tonsils probably are the common portal of entry of the organisms in rheumatism, and I may venture to say probably

a place where the organisms acquire the peculiar properties necessary to produce the disease known as rheumatism, other foci may be responsible, as shown by the following case:

A middle-aged man had a typical recurring attack of rheumatism one year after complete extirpation of the tonsils. (Other foci of infection, such as the prostate, were excluded.) The attack followed an indefinite intestinal disturbance. An organism resembling in every way, those obtained from the joints in the cases in which muscle

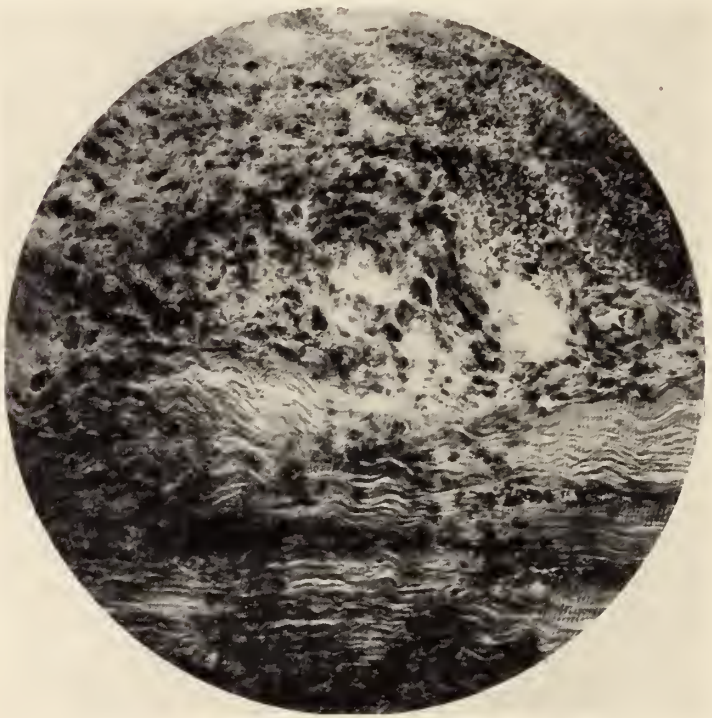

Fig. 1.—Microphotograph of human (biceps) muscle showing marked degeneration of muscle fibers leukocytic infiltration, and hemorrhage. Frozen section. Hematoxylin and eosin. ×230.

involvement was present was isolated from the flakes of mucus in the stools on three occasions. The patient was injected with a vaccine of the organism from other cases and with one prepared from the culture obtained from the feces. Recovery was rapid. After the joint symptoms had disappeared examination of the stool failed to show the coccus. The strain when isolated produced arthritis, pericarditis and endocarditis, while after one animal passage it acquired an affinity for the muscles, producing now myositis, myocarditis, endocarditis, and arthritis. Cultures from the blood during an attack of rheumatism in another case showed a similar organism.

The results of the cultures in the following case of muscular rheumatism are of special interest. Negro, laborer, 35 years old, admitted to the Cook County Hospital,

May 13, 1913, service of Dr. Patten. He had been ill for 12 days; illness began suddenly, after exposure to wet and cold, with severe pain and swelling first of left elbow and wrist joint. In a few days the knees became involved. When admitted the knees were still swollen and tender but soon improved. On May 17, the following notation was made: the day after admission the patient developed severe pain on the inner and upper aspect of right thigh. On May 15, there developed severe pain in the muscles of the neck and in the occipito-frontalis, associated with tenderness on pressure, and severe general muscular pain. On May 16, there developed sudden pain in lower third of the biceps of right arm and upper third of right forearm, with fever. Over these areas a distinct swelling was present.

The areas were cocainized and pieces of muscle removed. The muscles were hemorrhagic and edematous. The hemorrhagic areas were in close proximity to the tendinous parts but widely separated from the joints. Microscopic sections (see Fig. 1) revealed marked degeneration of muscle, extravasation of blood, and leukocytic infiltration. The patient was given large doses of a vaccine prepared from various strains from rheumatism and recovered without suppuration in the muscles and without developing further lesions.

Cultures were made by placing small pieces and emulsions of the muscle on the surface of blood agar slants and plates, dextrose-agar slants, Löffler's blood serum slants, into ascites-dextrose broth and into ascites-dextrose agar. The only growth on the surface of the various solid media and in the broth were three colonies of staphylococci, probably a contamination. The ascites-dextrose agar inoculated with material from the biceps gave 15 colonies, while from the forearm five colonies were obtained. These were all within a space 0.5 cm. from the free surface and 3 cm. from the bottom of the tube. The culture from the blood was made by getting rid of the hemoglobin and planting the sediment into ascites-dextrose agar. Fifteen colonies developed. This organism corresponds very closely indeed with those obtained from the joints in cases in which muscle involvement is present.

In two other cases of articular rheumatism in which distinct muscular involvement was present attempts were made to isolate the organism from excised pieces of muscle but without result, and sections did not show any lesions. The areolar tissue overlying a piece of the left suprascapular muscle in one case, however, showed a circumscribed area of leukocytic infiltration.

CHARACTERISTICS OF THE STREPTOCOCCI.

Three types of cocci have been obtained from the joints in rheumatism.

The strains from five cases, in none of which were muscles involved, produced green on blood agar, formed long chains of diplococci and large clumps in broth; when injected into animals they produced a nondestructive arthritis, pericarditis and endocarditis. The diplococci were distinctly larger and more uniform in size than those of the *Str. viridans*.

In six cases, all of which showed more or less muscular involvement in conjunction with arthritis, the organisms isolated produced

a slight but hazy hemolysis on blood agar plates, formed short chains and diplococci in broth, and when injected intravenously in rabbits commonly produced arthritis, endocarditis, some pericarditis, a myositis, and often a most pronounced myocarditis. They are the same size as hemolytic streptococci but the diplococcus arrangement in the chains is more marked.

In three cases, none of which showed symptoms referable to the muscles, the organisms produced small, grayish colonies on blood agar plates without perceptibly affecting the medium. They formed clumps of small micrococci and occasionally diplococci and short chains. One of these strains produced arthritis, endocarditis, and pericarditis but no muscle lesions.

These results would seem to explain why different observers have named organisms like these "*Streptococcus*," "*Diplococcus*," or "*Micrococcus rheumaticus*" depending on the particular type with which they happened to be working. The virulence of all three types is of a low order. The third group seems to be the least virulent, the first group occupying a middle position, those from cases of muscular and articular rheumatism being the most virulent. Tests of their susceptibility to phagocytosis place them in the same order. They are not bile soluble and they autolyse slowly in NaCl solution. There is no capsule.

A number of strains were lost in the first or second cultures, but they may live for a long time on blood agar and other media.

When first isolated they are all characterized by a marked capacity to multiply at a low temperature. They are very sensitive to oxygen pressure. Three of the strains of the first group and all of the strains which produced muscle lesions ferment mannite but not inulin and produce a high acidity in dextrose broth (5–7 per cent). This is particularly true of the strains which produce muscle lesions. They produce marked clouding on ascites-dextrose agar. At first they grow readily in filtrates of streptococcus broth cultures, whereas later they do not.

When injected intravenously in animals they produce lesions which correspond quite closely to those found in man.

After cultivation from one to eight months the capacity to grow at a low temperature, the sensitiveness to oxygen, the excessive

production of acid in dextrose broth, and the simultaneous affinity for joints, pericardium, endocardium, and myocardium are found to have largely or entirely disappeared.

For the details of experiments on mutation of these cocci reference is made to the paper on "Transmutations within the Streptococcus-Pneumococcus Group" (See p. 1).

Here the following statement may be made: By appropriate means strains of the three varieties have been converted each one into the other. Thus Strain 734 from articular rheumatism, produced green colonies on blood agar, long chains in broth and arthritis, endocarditis, and pericarditis in rabbits (see experiments, Rabbits 418, 374, 382, 356, etc.). After "soaking" the strain in distilled water for three weeks it produced a narrow zone of hemolysis on blood agar, diplococci and short chains in broth, and in addition to arthritis, endocarditis, and pericarditis produced also marked myositis and myocarditis just as the strains as isolated from cases of muscular and articular rheumatism (see experiments, Rabbit 428). After prolonged cultivation the strains which produced green at first come to resemble *Str. viridans* in morphology, in cultural, and pathogenic properties. The strains which produced a slight, hazy hemolysis at first now usually produced a wide zone of hemolysis, and when injected produced arthritis but no endocarditis and pericarditis. By means of animal passage strains of each group have been converted into typical pneumococci. This is most readily accomplished with the strains which produce the diplococci and short chains.

RECORDS OF EXPERIMENTS WITH STRAINS AS ISOLATED FROM ARTICULAR RHEUMATISM.

Rabbit 418:

March 5, 1913. Injected intravenously with the growth from 75 c.c. of ascites-dextrose broth of Strain 734. Death in 10 minutes. Large subendothelial hemorrhage in septum and papillary muscles of left ventricle. A few small hemorrhages in right auricle and on the cut surface of the myocardium. Circumscribed hemorrhages in the parietal pericardium. Fluids in the joints clear but cultures from one joint gave green-producing colonies of diplococci.

Rabbit 374:

February 11. Injected intravenously with the growth of 30 c.c. ascites-dextrose broth of Strain 734.

February 12. Dead. Two hemorrhages at base of pulmonary semilunar cusps and one near the free margin of adjoining cusps. Moderate amount of turbid fluid

in pericardium and joints. A number of hemorrhages in the periarticular structures, and in one joint a small hemorrhage in the intracapsular ligament. A few colonies of streptococci obtained from the blood, the pericardial fluid, and one joint.

Rabbit 382:

February 13. Injected in ear vein with the growth of 35 c.c. ascites-dextrose broth of Strain 734^{2}.* Died the next day. Multiple, embolic hemorrhage in mitral and tricuspid valves; small hemorrhages in myocardium and glomerular tufts. Mucous membrane of duodenum and of appendix hemorrhagic. Fluid from both knees contained a moderate number of leukocytes. A few colonies of streptococci obtained from blood, and from one joint.

Rabbit 356:

January 29. Injected intravenously with the growth from 25 c.c. ascites-dextrose broth of Strain 734 as isolated from the joint.

January 31. Lame, left front ankle joint swollen.

February 3. Dead. Pericardium distended with moderately turbid fluid with fibrin and leukocytes; fluids from left front ankle and other joints turbid. Smears from ankle joint showed a few diplococci. Two small subendothelial vegetations on the tricuspid valve. Cultures gave one green, nonadherent colony from blood, about 50 from pericardial fluid, and 150 from left ankle joint; no colonies from other three joints. The colonies from the joint fluid were quite different from those from blood and pericardial sac, being more elevated, less moist, surrounded by a narrow zone of hemolysis, and smears showed larger clumps of streptococci.

Rabbit 376:

February 11. Injected intravenously with the growth from 30 c.c. of ascites-dextrose broth of Strain 734^{3}.

February 14. Lame, right knee joint swollen.

February 17. Dead. No pericarditis, no visible myocarditis, small vegetation at apex of papillary muscle in left ventricle. Fluid from three joints turbid, from two other joints clear. Smears from the right knee joint showed large number of leukocytes, no reds, a few diplococci, mostly within cells. Cultures from blood, pericardial fluid, pelvis of kidney, and two joints sterile. Left knee joint gave a moderate number of green colonies.

Rabbit 378:

February 11. Injected intravenously with 5 c.c. of an emulsion in NaCl solution of a kidney from a guinea-pig which died immediately after an injection of Strain 734. The kidney was removed in a sterile manner and incubated for 24 hours in NaCl solution. A marked growth took place.

February 14. Marked swelling of left knee joint. Animal chloroformed. There were edema and numerous hemorrhages just where the muscles merge into the tendon sheaths and periosteum about the left knee. Smears from these areas showed many organisms. The joint fluid was turbid, containing many leukocytes, but smears showed only a small number of organisms. On opening the joint, hemorrhages were found in the intracapsular ligament and along the line of attachment of the ligaments. The opposite knee showed a similar condition except that the hemorrhages were less

* The figure to the right and above the number of the strain, indicates the number of animal passages.

marked, the joint fluid less in amount and less turbid. Three other joints were punctured but only one gave a turbid fluid. Cultures from the blood and fluid from three joints sterile. Large number of colonies from edematous and hemorrhagic areas, moderate number from fluid in left knee and few from right knee.

Rabbit 417:

March 5. Injected intravenously with the growth from 40 c.c. of ascites-dextrose broth of Strain 734[8].

March 7. Injection repeated.

March 8. Dead. Hemorrhages about joints; most marked about knee joints; intracapsular ligament of right knee showed two small hemorrhages; joint fluids turbid; small round subendothelial, grayish white, nodules in tricuspid valve; pericardial sac contained a moderate amount of slightly turbid fluid; small punctate hemorrhages in cortex of kidney; small punctate scleral hemorrhages in the limbus; small nodules in iris. Smears from joint fluid showed a few, while those from the hemorrhagic areas showed a larger number of diplococci. Cultures from blood and knee joints gave a few colonies while the bloody fluid around joints and the nodules in iris gave large number of green-producing colonies of gram-staining diplococci and chains.

RECORDS OF EXPERIMENTS WITH STRAINS FROM CASES OF ARTICULAR AND MUSCULAR RHEUMATISM AND WITH OTHER STRAINS WHICH HAVE BEEN MADE TO RESEMBLE THESE.

Rabbit 418:

March 10. Injected intravenously with growth from 45 c.c. ascites-dextrose broth of Strain 734 after it was made to resemble the strains from muscular rheumatism.

March 12. Dead. Numerous, elongated grayish white areas, 2–10 by 1–2 mm., running parallel with the muscle fibers in the skeletal muscles, especially in the intercostal muscles, the flat muscles of the abdomen (see Fig. 2), neck and shoulder, in the muscles of the back and in the more tendinous portions of the muscles of the extremities and the diaphragm. A small number were found in the upper end of the esophagus, the muscles about the larynx, but only one in each of the masseters. Smears from these areas in the muscles showed a moderate number of gram-staining diplococci, and leukocytes. The character of these lesions is illustrated in Figure 3. Such lesions were not found in the muscular coat of the stomach, or intestines, but several small grayish white nodules were found in the wall of the bladder. Small numbers of grayish white but less circumscribed and more irregular areas were found in the myocardium of the right ventricle, but not in the left ventricle, and there were small subendothelial grayish white nodules in the tricuspid valve and chordaetendineae. No hemorrhages about joints but the fluid in three of five joints was turbid. The mucous membrane of the appendix showed marked hyperemia with a number of small ulcers. The kidney showed whitish, linear areas in the medullary portion; cortex normal. At the apex of one of the areas there was a small ulcer in the pelvis of kidney, the smears showing diplococci, short chains and leukocytes. The mucous membrane of the pelvis of the kidneys appeared edematous. The urine contained a large number of leukocytes, columnar epithelial cells, and some diplococci and short chains. Cultures on blood agar plates gave a small number of slightly hemolysing colonies from the blood, one joint, and piece of crushed muscle not containing visible lesions, while cultures from the other turbid joint fluid, the crushed vegetation, pelvis of kidney, whitish area in

medullary portion of kidney, the urine, and piece of muscle containing one of the grayish streaks showed a large number of similar colonies.

Dog 25:

March 26. Injected into vein of hind leg with the growth of 240 c.c. of ascites-dextrose broth of Strain R 51A[20]. This strain, originally a pneumococcus, was made to resemble the strains from muscular rheumatism.

FIG. 2.—Photograph of lesions in abdominal wall in Rabbit 418 (see protocol). ×5.6.

March 27. Lame in left front leg, weakness in extremities.

March 28. Turbid fluid from both knee joints. Cultures gave a few very small slightly hemolysing colonies.

March 31. Right front wrist swollen, fluid turbid. Very tender over muscles of back and neck. Pressure here and over the tendinous portion of muscles of extremi-

ties caused animal to yelp, whereas same amount of pressure over belly or muscles of extremities did not. Marked tenderness in groin. Animal chloroformed and muscles exposed. A number of grayish white areas similar to those in rabbits were found and two were excised. Cultures gave streptococci and sections showed inflammatory areas.

April 3. No lameness but animal seemed weak; walked about very little, ate little, and drank water only.

April 8. Found dead, body still warm. Tissues everywhere pale. A large number of grayish white streaks in muscles about neck, shoulder, under shoulder

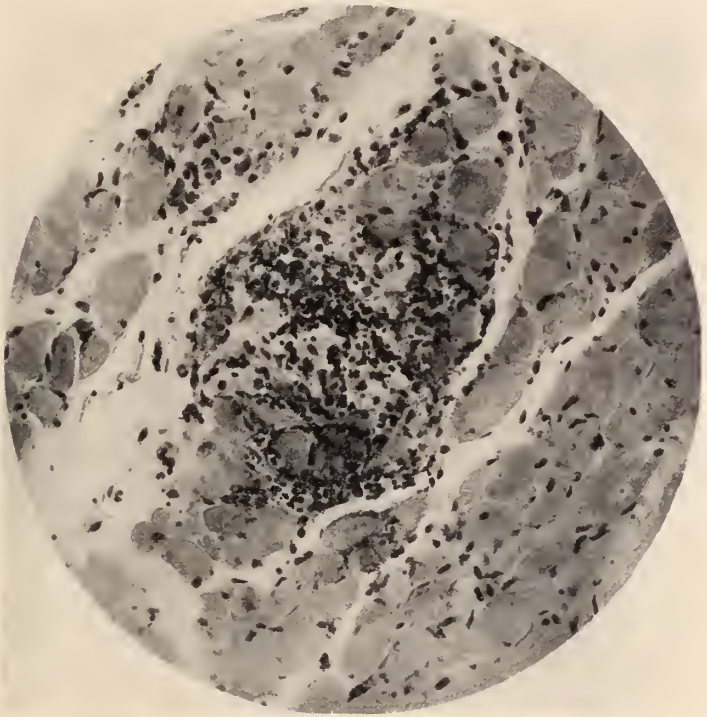

FIG. 3.—Microphotograph of lesion in muscle (trapezius) of Rabbit 418. Death 48 hours after injection. Note degeneration of muscle fibers, leukocytic, and round cell infiltration. Hematoxylin and eosin. ×230.

blade, intercostal muscles, diaphragm, and the more tendinous portion of the muscles of the extremities. One distinct abscess about shoulder joint. Joint fluids clear. Myocardium grayish red and flabby. A number of whitish areas were found in the wall of the right ventricle and in the apex of the left papillary muscle. Pericardium and endocardium normal. Kidneys showed yellowish white linear areas radiating from pelvis where there were a number of small ulcers. Pelvic mucous membrane was edematous. Urine contained moderate number of leukocytes and columnar epithelium. Large, deep ulcer of duodenum (1×0.5 cm.) two centimeters beyond

pyloric ring. The margins were undermined and infiltrated. Local peritonitis with adhesions to omentum; another, similar ulcer three centimeters from pyloric ring. Intestines contained large amount of partially digested dark red blood. Thyroid on cross section showed several whitish areas similar to those in the muscles. Liver showed a moderate fatty degeneration. The gall bladder was filled with bile containing mucus and flakes of pus. The lungs, adrenals, appendix, meninges and brain showed no gross changes. Cultures on blood agar plates from blood, joints, and pericardial fluids were sterile; from pelvis of kidney, white streak in medullary portion, urine and pus from about shoulder joint gave moderate number, while the bile showed a very large number of slightly hemolysing streptococci in pure growth. Sections from the ulcer in the duodeum, stained by Gram-Weigert's method showed a moderate number of deeply staining diplococci in the wall of the ulcer.

Monkey:

March 18. Leukocytes, 10,500. Injected with the growth from 100 c.c. of ascites-dextrose broth culture of Strain 734H into vein of forearm.

March 20. Leukocytes, 24,500. Seemed quite well. Both knee joints aspirated and cultures made. Injection repeated.

March 22. Cultures from one joint sterile, those from the other showed eight green-producing colonies. Leukocytes, 9,400. Seemed weak. Anesthetized and a small portion of trapezius excised.

March 24. Suppurative conjunctivitis in left eye. Cultures gave a few green-producing colonies as was also the case with cultures from knee joint.

March 25. Leukocytes, 24,800.

March 31. Seemed very sick; severe diarrhea; took no food.

April 2. Dead. Moderate number of grayish white lesions in intercostal muscles anteriorly, in flat muscles of the groin, in scapular and spinal muscles, in intervertebral and tendinous portion of muscles of extremities. These areas were quite similar to those found in rabbits and dogs. Smears from the areas showed leukocytes but no bacteria. Pericardium contained a small amount of turbid fluid. In the epicardium there were a number of linear scars which joined the right auricle and right ventricle, and inclosed a gelatinous, grayish yellow material, smears of which showed leukocytes and an occasional gram-staining diplococcus. A number of subendothelial, whitish nodules in apices of papillary muscles and posterior leaflet of both mitral and tricuspid valves. Myocardium grayish red and soft. Stomach normal except for the presence of three deep ulcers, two near the pyloric end, the other and largest being in the pyloric ring. All had a clean base, were undermined, and had infiltrated margins. The largest had bridges of mucous membrane over undermined portions. Omentum was adherent to stomach and in the largest ulcer there was a small perforation of the serous coat. Small intestines normal. The mucous membrane of the large intestine was hyperemic. The lymph follicles were swollen and a number showed superficial ulceration; a number of these ulcers appeared directly opposite whitish areas in the serous coat. The lymph glands in the mesentery and about the pyloric end of stomach were enlarged and hyperemic. Joint fluids clear. No lesions of brain and cord. The sheath of sciatic nerve contained a large number of very small, whitish areas. Cultures on blood agar plate showed blood and joints to be sterile; the muscle areas and gelatinous material from epicardium yielded a few colonies of streptococci; lymph glands showed mostly colon bacilli, but a few slightly hemolysing colonies; the material in one ulcer in stomach showed saprophytes and colon

bacilli only, but from a small, thoroughly washed, and crushed piece of the wall of the ulcer there were obtained, in addition to contaminating organisms, 15 colonies of streptococci. The healthy mucous membrane showed no streptococci but contaminating colon bacilli only.

GENERAL SUMMARY OF THE ANIMAL EXPERIMENTS.

The muscle lesions have been produced in numerous rabbits, dogs, and one monkey. The character of the lesions was similar in all. The number of lesions varied from three to many hundred. In no instance did they lead to suppuration. The lesions have been obtained with four strains as isolated from joints in cases of rheumatism in which muscular involvement was present, with a strain from articular rheumatism after having been modified, and with six strains of "non-virulent" streptococci after they were made to correspond morphologically, culturally (especially on blood agar and ascites-dextrose agar), in resistance to phagocytosis and virulence, to the strains from cases of muscular and articular rheumatism in man. The localization in the muscles of the "non-virulent" strains of streptococci occurred only after from 12 to 21 animal passages. When the various strains produced myositis their affinity for the muscles was so marked that each of a series of animals when injected with the same strain, not only developed the lesions, but the number was quite in proportion to the size of the dose injected. After one or two animal passages this affinity disappeared and it was impossible again to produce the muscle lesions. Owing to the fact that the grade of virulence of the organisms is such that they tend to disappear from the general circulation it was possible to study the exact relation of the organism to the lesions produced. As illustrated by the experiments, streptococci have been found with leukocytes in smears from the lesions and the former have been shown to be alive by cultures, smears and cultures from adjacent muscle either being entirely negative or showing a much smaller number of bacteria. Gram-staining diplococci have been found in sections. The organisms in the muscle lesions were most numerous 48 hours after injection. Small linear scars were formed eventually.

A study of the sections of muscles with lesions shows that there is produced first a small hemorrhage. The muscle fibers then

lose their striations, stain poorly, become granular and break up into fragments as a rather sharp leukocytic infiltration appears (see Fig. 3). The number of organisms is greatest at this time, after which they gradually disappear without causing suppuration. The leukocytes now give way to larger mononuclear cells and as connective tissue is being formed there is found a deeply basic

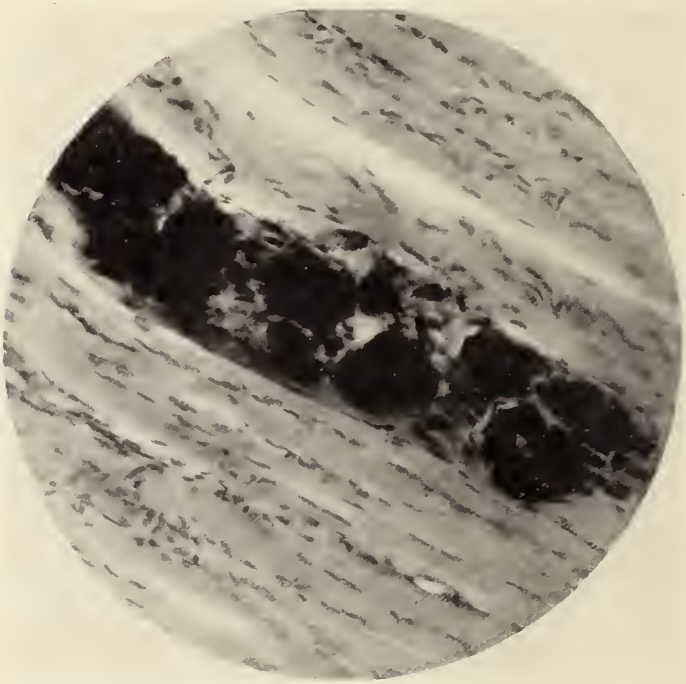

Fig. 4.—Microphotograph of lesion in muscle of dog. Death two weeks after injection. Healing stage. Note the almost complete absence of leukocytes. Hematoxylin and eosin. ×230.

staining material in which bacteria are no longer demonstrable (Fig. 4).

Thirty-five rabbits were injected with strains from rheumatism. Seven of the 10 which died within 24 hours showed subendothelial hemorrhages of heart valves or papillary muscles. The valvular hemorrhages were smaller than those following injections of *Str. viridans*. They occurred most often in the following order: tricuspid, mitral and base of pulmonary and aortic cusps. Four showed myocardial hemorrhages, five pericardial hemorrhages, and six periarticular hemorrhages. Similar hemorrhages were observed in the rabbits which died soon after a second or third

injection. Endocarditis was observed in 20 out of the remaining 25 rabbits. The tricuspid valve alone was involved 11 times, the mitral alone four times, the aortic alone twice, both tricuspid and mitral endocarditis twice, and mitral and aortic endocarditis once. The vegetations were nearly always small subendothelial, grayish nodules which in the early stages were quite soft, smears now showing diplococci and leukocytes, and cultures, relatively large numbers of organisms. Later they became glistening white and quite firm, smears now no longer showing leukocytes nor bacteria. Healed endocarditis of the tricuspid valve was observed twice.

After animal passage or on otherwise modifying the strains large vegetations have been obtained which resemble those following injections of *Str. viridans*. The mode of origin of the experimental endocarditis is an embolic process just as in endocarditis due to *Str. viridans*. Myocarditis which was easily recognizable was observed eight times and almost without exception after the injection of strains which produced the lesions in the skeletal muscles. Acute pericarditis has been observed 11 times. Localized adhesive pericarditis twice. Pus was never found. The number of organisms in the exudate was always small. Adhesive pleuritis has been found once. In the 25 animals which lived long enough, arthritis was found 18 times. This was usually multiple, never suppurative, and the number of organisms was never very large. The tendency to healing was marked. The number of organisms was larger in the joints when the animals were injected with strains which produced muscle lesions. Endocarditis, pericarditis, myocarditis, and arthritis were observed six times in the same animal; endocarditis, pericarditis, and arthritis five times; endocarditis and arthritis six times; acute hemorrhagic or ulcerative appendicitis was observed five times. Suppurative appendicitis has not been observed. Acute hemorrhagic mesenteric lymphadenitis is very common following intravenous injections of these organisms. Scleral hemorrhages chiefly in the limbus occurred 10 times, in the retina twice and in the iris three times. Iritis, due to the organisms injected, has been observed twice. The ulcers of the stomach and the renal lesions will be described in separate papers. It should be said here that the strains from articular rheumatism which did not produce muscular lesions rarely produced a recognizable infection in the kidney, altho punctate hemorrhages in the cortex were observed in the animals which died in 24 to 48 hours after injection. The results following injections of the strains which produced muscle lesions were strikingly different. They produced hemorrhages in the medullary portion and pelvis of the kidney in the animals which died early, and almost constantly the picture of an "ascending nephritis" in both dogs and rabbits which died later.

Five rabbits (and two rats) all of which were injected either with the strains from rheumatism after one animal passage or with one strain of a streptococcus which had similar properties, showed symptoms of meningeal irritation during life. Two of the rabbits which died within 36 hours after injection showed small circumscribed meningeal hemorrhages. The other three rabbits and one rat which died spontaneously or which were chloroformed, five to 20 days after injection, showed localized grayish white nodular areas in the pia mater. Cultures from the crushed areas yielded the organism in pure culture in two of the rabbits. Cultures from the blood proved negative. Gross lesions in the brain substance have not been found but sections showed subcortical areas of round cell infiltration in two. The spleen in the animals injected with the strains from rheumatism as isolated has never been enlarged.

EFFECT OF COLD ON RHEUMATISM.

It is a well-known fact that exposure to cold aggravates the symptoms in rheumatism. The results of the work of Poynton and Paine, of Beattie, and of myself show that this is true also in experimental rheumatism. As far as can be determined, however, no explanation has been advanced of this action of cold. Benedict and Slack[1] in a study on the fluctuations of temperature in different parts of the human body show that variations of from one to two or three degrees Centigrade are common. It occurred to me that the aggravation of symptoms in rheumatism might be due in part to the ability of these organisms to grow well at a low temperature as shown by the following experiments:

Test tubes 20 cm. in length with a caliber of 8 mm. were used. Long slants of ascites-dextrose agar covering the whole length of the tube were made and inoculated on the surface. The lower end was placed in running cold water and the upper end into a thermostat at 48° C. A control tube containing a thermometer to determine the temperature at the different levels was also included. The growth of six strains of pneumococci, three of hemolytic streptococci obtained from various sources, and seven strains of streptococci from rheumatism was studied. The average limit of growth of the pneumococci was 12 cm. from the bottom, at a temperature of approximately 32° C., of the hemolytic streptococci 8 cm., at a temperature of approximately 27° C., and of the strains of streptococci from rheumatism 6.5 cm., at a temperature of 22° C. The average point of optimum growth of the pneumococci was 18 cm. from the bottom, at a temperature of 38° C., of the hemolytic streptococci 16 cm., at a temperature of 37° C., and of the streptococci from rheumatism 12.5 cm., at a temperature of 35° C. Blood agar slants gave similar results. The ability, therefore, of the strains from rheumatism to grow at a low temperature is striking.

Frogs were inoculated with comparable doses of virulent pneumococci, hemolytic streptococci, and streptococci from rheumatism. Three frogs were inoculated intraperitoneally with each strain and three sets of experiments in which the frogs were kept at from 22° C. to 25° C. were made. Smears and blood agar plate cultures were made at intervals after inoculation. The results in the three sets of experiments were similar. The frogs receiving virulent pneumococci remained well and the organisms disappeared from the peritoneal cavity and blood. Three of the nine frogs injected with hemolytic streptococci died in two, three, and six days respectively. All of the frogs injected with streptococci from rheumatism died in from two to seven days from streptococcemia. The order of "virulence" of these strains here is exactly the reverse of that in warm-blooded animals. Knowing that the frog's temperature is that of its surroundings, similar experiments were made (except that approximately one-twentieth of the dose was given) in which the frogs were kept at 37° C. They were supplied with water and the incubator was well ventilated. Here the order of virulence was the same as for warm-blooded animals, the pneumococci killing most promptly, then

[1] *Publication No. 155, Carnegie Institution*, 1911.

the hemolytic streptococci, while those injected with streptococcus from rheumatism survived entirely or died latest. The control frogs lived indefinitely under the same conditions.

In connection with these experiments the following observation was made: The amount of phagocytosis in the smears from the blood and peritoneal exudate of the frogs injected with virulent pneumococci was strikingly greater in the experiments at a lower temperature than in the ones at 37° C., whereas in the case of the rheumatic streptococcus the reverse was true. This fact may be an additional reason why exposure to cold aggravates symptoms in rheumatism and suggests that the resistance or nonresistance to phagocytosis of bacteria may depend to a certain degree on the activity of growth—"growth pressure."

A board, in which two slits were sawed out at one end, was well padded and oiled with paraffin oil. A medium-sized rabbit (No. 457) was injected in the ear vein with the growth from 15 c.c. of rheumatism, Strain 735. It was now fastened to the padded board with a towel in such a way that the hind legs extended through the slits in the board. The right was placed in running water, the left kept at room temperature. The animal died the next day. The muscles and structures around the joints of the right leg showed numerous hemorrhages. No hemorrhages could be found above the line of exposure to cold and only a few in the opposite leg. The right knee and ankle joints gave a large number of leukocytes and many colonies while those from the left gave only a few. The blood contained a few bacteria.

The reasons then why exposure to cold aggravates the symptoms in rheumatism may be that the exposure tends to lower the temperature of the part directly and to cause vasomotor constriction; the lowered temperature would favor the growth of the organisms directly, probably diminishing the activity of the leukocytes, and possibly increasing resistance to phagocytosis because of more active growth.

The results of cultures from the joints show that the organisms as found in man are very sensitive to oxygen pressure. Hence vasomotor constriction besides tending to lower the temperature might favor the growth of the organisms by causing a lack of blood and leukocytes and in consequence a lowered oxygen pressure. Judging from experiments which I have made on the production of toxic substances during autolysis of pneumococci under various degrees of oxygen tension and from the results of Amberg and

Knox,[1] lack of oxygen in addition to favoring the growth of the organisms in rheumatism might increase markedly the amount of toxic substances produced during their disintegration; at the same time the ischemia from vasomotor constriction would tend to increase the concentration of the toxic substances.

CONCLUSIONS.

Three types of organisms have been isolated from the joints in rheumatism, each of which can be converted into the other quite readily.

Two types, one a very long chain producer, the other resembling a micrococcus, have been obtained from cases in which no muscle involvement was present. When injected into animals as isolated they commonly produce arthritis, endocarditis, and pericarditis, but not usually a visible myocarditis, and never a myositis.

The third type, a diplococcus with short chains of diplococci, was obtained from cases of rheumatism in which definite muscular involvement was present. It also produces arthritis, endocarditis, and pericarditis, but especially, marked myocarditis and myositis.

All three forms which have a low grade of virulence are quite freely susceptible to phagocytosis.

The results of the cultures in man and of the animal experiments support the view that acute articular rheumatism is due to streptococci which have peculiar properties.

Experimental and other evidence has been produced to indicate that the more or less closely related condition, muscular rheumatism or "rheumatic myositis," is due to streptococci, closely related to those in articular rheumatism.

The name *Str. rheumaticus* may be retained at present, not with the idea that the organisms so called always produce rheumatism, but rather to call attention to the fact that when streptococci produce the symptoms and lesions of rheumatism they have certain special features which streptococci from other sources do not usually have.

The affinity for joints, endocardium, pericardium, and often also myocardium and muscles which characterizes these organisms

[1] *Jour. Pharm. and Exp. Therap.*, 1912, 31, p. 223.

when first isolated, tends to disappear on cultivation. It may be restored by animal passage, and other strains of streptococci under certain conditions may be made to acquire the features of the strains from rheumatism. When the rheumatic strains have acquired the cultural features of hemolytic streptococci they lose the affinity for the endocardium and pericardium and acquire an even greater affinity for the joints. When they have been converted into pneumococci of a certain grade of virulence pulmonary hemorrhages and pneumonia are commonly found after intravenous injections whereas when the virulence is still greater death from pneumococcemia results. These and other facts suggest strongly the possibility that previous to an attack of rheumatism various types of the streptococcus group, especially hemolytic streptococci, acquire in the tissues of the infected individual the features which give them the simultaneous affinity for joints, endocardium, pericardium, and myocardium.

The experiments on mutation show that when these and other streptococci are grown in symbiosis with other bacteria, and under a low oxygen pressure they may acquire new features, and that sometimes they undergo marked changes on passage through animals. The places in the human body where such conditions prevail and where special features are likely to be acquired are parts of infection such as in the tonsils, various sinuses, the appendix, and about the gums and teeth. That this actually occurs in the tonsils in rheumatism seems quite clear; the mild character of the tonsillitis at the time of the attack and the late appearance of rheumatism in some cases of acute follicular (streptococcal) tonsillitis accord with this idea. The importance of focal infections as a point of entrance of bacteria in general is quite well recognized, but the idea that the focus serves in addition as a place where bacteria can acquire new properties is not generally recognized and needs to be emphasized.

Experimental evidence has been produced to show that probably lodgment of organisms in the fine capillaries of the iris occurs in rheumatic iritis. The lesions in the appendix, the diarrhea due to colitis, and the enlargement of the mesenteric lymph glands observed commonly in animals after intravenous injections of these

strains, as well as their isolation from the stool during rheumatism in man, indicate that the organisms may gain entrance through the lymph structures of the intestinal tract.

The strains from muscular rheumatism, especially after one or two animal passages, as well as other streptococci when they have attained a similar grade of virulence, show a marked affinity for the mucous membrane of the stomach, the pelvic mucous membrane and medullary portion of the kidney and the gall bladder. Ulcer of the stomach, the picture of an "ascending" nephritis, cholocystitis with beginning formation of gall stones, caused by streptococci, have been found repeatedly in rabbits and dogs injected with these strains, especially after one or more animal passages.

OBSERVATIONS ON INDOL PRODUCTION BY BACTERIA OF THE COLON-TYPHOID GROUP.*

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While the production of indol in a peptone solution is extensively used as a test for certain groups of bacteria, most authors are rather skeptical as to its real classificatory value. Many variable results have been obtained by different workers, as a result of which the test has been greatly discredited. Nevertheless it is still used in most laboratories.

Recently Zipfel[1] suggested the use of tryptophan, the mother substance of indol (indol-a-amino-propionic acid), for testing the power of bacteria to produce indol. In a series of studies on the behavior of a large number of bacteria of different groups toward that substance, he obtained strikingly constant results. The organisms of the same group either all do or do not produce indol and generally in 24 hours. Seidelin,[2] however, showed that while bacteria were capable of producing indol from tryptophan, at no time during his tests could he demonstrate the presence of tryptophan in a peptone water culture of an indol-producing organism. Indol is produced from peptone and tryptophan, therefore, apparently by two distinct processes, and while the latter may be a useful test, it cannot take the place of the peptone test. Besides, tryptophan is so difficult either to prepare or obtain that its extensive use is out of the question at present.

In the present state of bacteriological technic, many reasons other than the variability of the reaction may be found to explain the inconstancy of a certain test. The technic may differ in several respects. Very often different methods are employed in preparing the peptone solution. Some use distilled water, others tap water, and still others add salt either to the distilled or to the tap

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[1] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1912, 64, p. 65; *ibid.*, 67, p. 572.

[2] *Jour. Hyg.*, 1911, 11, p. 503.

water solution. While these variations will not affect the actual production of indol they may affect the time in which sufficient indol is produced to respond to the test. Another factor is the mode of inoculation of the medium. The age and amount of the culture inoculated will, of course, influence the course of indol production. Finally the time of incubation varies widely; some employ 4, others 6, and still others 10 days for incubation. It is evident that the above factors when combined may have a decided influence on the recorded result.

The method used for making the test may also constitute a very serious source of error. In America the Salkowski test (H_2SO_4+ KNO_2) is widely used and recommended by the Committee on Standard Methods (1912) as a test for indol. Böhme (1905)[1] and Marshall (1907)[2] after a series of comparative tests concluded that the Ehrlich method (paradimethylamino-benzaldehyde+HCl) is more sensitive and gives more constant results. MacConkey (1909)[3] claims that while the Salkowski test does give variable results, the Ehrlich test rarely varies.

While conducting a series of tests on certain members of the colon-typhoid group, I thought it desirable to ascertain the constancy of this reaction when the factors enumerated above are carefully controlled. The test was performed on the same series of organisms on three different occasions, using different incubation periods but employing both the Salkowski and the Erhlich tests. In this way the constancy of the organism, the importance of the incubation period, and the relative value of the two tests were determined.

The technic employed was in brief as follows: a peptone solution consisting of H_2O (distilled), 1000 c.c.; peptone (Witte's), 10.0 gm.; K_2HPO, 0.2 gm.; NaCL, 5.0 gm. was made up, filtered through filter paper, tubed, 10 c.c. to a tube, and autoclaved. This medium gave excellent growth.

Each culture to be tested was inoculated into a tube of this peptone broth and incubated for 24 hours. The 24-hour culture was then used for the inoculation of the peptone broth to be tested. One cubic centimeter of the peptone culture was inoculated into each of 5 tubes of broth by means of a sterile pipette, and these were incubated at 37°C. Periods of 2, 4, and 6 days respectively were employed for incubation, the tests being performed at approximately monthly intervals. In all of these cases the Ehrlich method was used.

[1] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1905, 40, p. 129.

[2] *Jour. Hyg.*, 1907, 7, p. 581.

[3] *Jour. Hyg.*, 1909, 9, p. 86

For the determination of the relative value of the two tests, duplicate peptone broth tubes of each culture were incubated for 4 days. The contents of each tube were then divided into two parts, and subjected to the Ehrlich and Salkowski tests respectively. The same tube was thus tested by both methods. The rest of the technic was the same as above.

The Ehrlich test was made by adding 1 c.c. of a 2 per cent solution of the aldehyde in 95 per cent alcohol and then adding concentrated HCl drop by drop until a red zone appeared between the alcohol and peptone layers. Not more than 0.5 c.c. of acid is necessary. On standing for about 15 minutes the red zone deepens and forms a wider ring. Each tube was then shaken up with chloroform and when it dissolved the red color the test was considered positive. By using approximately the same amount of chloroform for each tube, an idea of the relative amount of indol formed can be obtained from the intensity of the color.

The Salkowski test was performed in the usual way by adding first 1 c.c. of 10 per cent H_2SO_4 and then slowly 1 c.c. of 0.01 per cent solution of potassium nitrite. A pink to red ring is thus formed which deepens on standing. In a few cases the whole tube reddened almost instantly, but on shaking with chloroform none of the color went into solution. This phenomenon will be referred to later.

In all, 75 cultures were tested, falling roughly into the following groups:

B. communis	13	*B. communior*	13
B. aerogenes, *B. acidi-lactici*	20	*B. cloacae*	9
B. proteus	5	*Paratyphi group*	10

The results obtained are summarized in the tables below:

TABLE 1.

EHRLICH METHOD.

GROUP	NO. OF CULTURES TESTED	INCUBATION PERIOD		
		2 Days	4 Days	6 Days
		No. of Positive Results	No. of Positive Results	No. of Positive Results
Communior	13	10	10	10
Communis	13	12	12	13
Aerogenes-lactici	20	6	8	8
Cloacae	9	0	0	0
Proteus	5	3	3	3
Paratyphi	10	0	0	0

Table 1 points out the constancy with which the organisms react when the test for indol is carried out according to the Ehrlich method. In the communior group the same 10 organisms were repeatedly positive while 3 were negative. All of the communis

organisms were repeatedly positive and in all duplicate tubes. Twenty-three out of 26 colon organisms, or 90 per cent, were thus repeatedly positive. This is all that can be expected and the colon bacillus is justly called indol positive.

Only 8 out of 20, or 40 per cent, of the aerogenes organisms are indol positive. With these weaker indol-producers 2 days' incubation is not sufficient for the test. On the whole the 4-day period is satisfactory.

All the cloacae and paratyphi are repeatedly negative, while the proteus cultures divide into two groups—one indol positive, the other indol negative.

In all cases all the tubes used for each organism reacted alike.

TABLE 2.

INCUBATION PERIOD—4 DAYS.

GROUP	NO. OF CULTURES TESTED	POSITIVE RESULTS OBTAINED WITH	
		Ehrlich Test	Salkowski Test
Communior	13	10	10
Communis	13	12	11
Aerogenes-lactici	20	8	8
Cloacae	9	0	2
Proteus	5	3	5
Paratyphi	10	0	1

Table 2 gives a comparison between the two methods. The Salkowski results are higher as was also noted by Marshall (1907).[1] Besides this there were five instances in which the Salkowski test gave positive results in one tube and negative in the other. These tests were counted positive for convenience, because both of the Ehrlich tubes of the same strain were positive. These aberrant results illustrate one of the possibilities of error. I am inclined to think that the negative Salkowski tests in this case were perhaps due to a rapid oxidation of the red coloring matter.

The high results obtained with the Salkowski test are attributable to the formation of a red color which apparently is not due to indol. If the test is carefully performed this reddening can be distinguished by its rapid diffusion throughout the tube from the

[1] *Loc. cit.*

red ring obtained with indol. This reaction was obtained in all those strains in which the Ehrlich test was negative and the Salkowski positive. A similar reaction was obtained with a non-indol producing spore-former. In none of these cases was the color soluble in chloroform. Here then we evidently have a substance which gives a similar tho not the same reaction as indol and which can easily be confused with it. The results indicate that the Ehrlich test is to be preferred to the Salkowski.

An interesting phenomenon pointed out by Seidelin and Lewis (1911)[1] was also observed by me in connection with the Ehrlich test. This consists in the formation of a purple to bluish color which is insoluble in chloroform. Lewis claims that three distinct reactions are obtained: (1) soluble+insoluble red; (2) soluble red+purple or blue; (3) no soluble red, but insoluble blue. The soluble red observed by Lewis is, of course, the indol red. I have not met with any insoluble red pigment in my tests with the Ehrlich method. The purple color apparently is but a primary oxidation stage of the blue and both appear to be independent of indol production as indicated by the following observations:

1. If the tubes are shaken up with chloroform without the previous addition of persulfate the supernatant liquid is colorless. On standing for some time the liquid gradually assumes a purplish and eventually either blue or purple-blue color. This was observed in all cases. Often of two duplicate tubes one was purplish blue, the other blue.

2. The addition of a few drops of fuming HNO_3 or H_2O_2 (oxidizing agents) to the decanted supernatant liquid produced instantly the same changes of color observed on long standing.

3. The blue color reaction was obtained in uninoculated controls and also in a solution of peptone in distilled water treated with the aldehyde and hydrochloric acid. This shows that this reaction is independent of indol production. Since the aldehyde plus concentrated HCl alone does not give this reaction, it is evident that the blue coloration is due to the peptone. Whether it is specific for peptone or whether it is caused by one of the substances present in Witte's peptone mixtures still remains to be determined.

[1] *Loc. cit.*

CONCLUSIONS.

From the study presented above it appears:

1. That the indol reaction is sufficiently constant to be of diagnostic value.

2. That the Ehrlich test is constant and more reliable than the Salkowski. The test should be made on the fourth or sixth day and the tubes should always be shaken up with chloroform as a confirmatory test.

3. That the Salkowski test is unreliable because a red coloration is frequently obtained which is not due to indol, but to another substance and may be mistaken for it; and because the reaction in cultures which really produce indol is not constant.

4. That with the Ehrlich test *B. coli* is generally indol positive (+); *B. aerogenes* and *B. proteus*, variable (±); and *B. cloacae* and *B. paratyphi*, always negative (−).

5. That the blue color obtained in connection with the Ehrlich test is entirely independent of the indol test and is due to some substance present in the peptone.

STUDIES ON THE CULTIVATION OF THE VIRUS OF VACCINIA. II.*

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Two years ago[1] we began to apply the method of cultivating tissues *in vitro* (Harrison) to the study of the viruses of certain infections in which the specific living agents have not yet been identified. The three viruses studied thus far with the new method have been those of rabies, vaccinia, and syphilis.[2] In the study of the former we attempted, first, to determine whether the Negri bodies, the nature of which is still in dispute, would show any development or multiplication *in vitro* when fragments of brain from rabid animals were incubated in blood plasma, and, second, to see if the characteristic bodies could be produced *in vitro* by combining normal brain and virus. We found that no development or multiplication took place *in vitro*, and that, altho structures indistinguishable from certain forms of Negri bodies developed in the virus-normal brain preparations, the same bodies were found in control preparations in which were pieces of normal brain without virus. In a single instance the virus remained alive after 8 days' incubation; animal inoculations of preparations incubated for a longer time gave in all cases negative results.

During the past year we have been applying Harrison's method to the cultivation and study of the virus of vaccinia. The results of our first series of studies appeared recently in this Journal.[3] We concluded that there was a definite multiplication of the virus in tissue cultures composed of blood plasma and pieces of cornea (of rabbit or guinea-pig) to which small quantities of diluted virus were added. Successful animal inoculations were obtained from the third subculture. Histological studies of the preparations,

* Received for publication November 3, 1913.

[1] Steinhardt, Poor, and Lambert, *Jour. Infect. Dis.*, 1912, 11, p. 459.

[2] Steinhardt, *Jour. Am. Med. Assn.*, 1913, 61, p. 1810.
Steinhardt, Israeli, and Lambert, *Jour. Infect. Dis.*, 1913, 13, p. 294.

however, revealed no specific vaccine bodies. The present paper is concerned with the continuation of these experiments.

Levaditi[1] has recently applied Harrison's method with some success to the cultivation of the virus of poliomyelitis.

We wish to record at this time experiments upon the following points: (1) estimation of the extent of multiplication of the virus in cultures by use of higher dilutions of original virus; (2) necessity of *living* corneal tissue for the growth of the virus; (3) possibility of substituting other organ tissues for cornea; (4) use of plasma and cornea from immune animals in the culture preparations.

USE OF HIGHER DILUTIONS OF VIRUS.

In previous experiments, described in our first paper,[2] the virus was diluted 1:5 before use in the cultures. Inoculation of a small number of unincubated preparations in the skin of rabbits gave in some instances 50–60 pustules; the incubated preparations, on the other hand, produced such extensive confluent eruptions that the number of pustules could not be estimated with accuracy. In order to determine the extent of multiplication of the virus, it therefore seemed desirable to make use of a higher dilution of the virus. With this end in view the following experiments were carried out:

Experiment 501.—Two commercial glycerinated viruses (2190 and 2193) were dialyzed in salt solution according to the method of Poor and Steinhardt[3] and placed in icebox to allow coarse particles to sediment. Only the supernatant fluid was used.

Virus 2190 was diluted 1:15 in Ringer's solution. A large number of preparations were made with rabbit cornea and plasma and the diluted virus, using the same technic as described in our first paper. Nine unincubated preparations inoculated in the skin of a rabbit gave 10 flat pustules (sixth day). It should be mentioned that with these preparations a very dilute virus gives frequently flat pustules. This may be due to a lytic action of plasma on virus. The same number of slides, incubated 11 days, gave upon inoculation 60–65 elevated pustules. Practically all of the preparations in this series showed contamination with bacteria, and, consequently, only a slight and transient growth of corneal epithelium. Whether or not bacteria interfere with the growth of the virus in tissue cultures, we are not yet prepared to say, since contaminations have been exceptional in our experiments. In this instance the virus was probably the source of the infection.

[1] *Compt. rend. Soc. de biol.*, 1913, 74, p. 1794.

[2] *Jour. Infect. Dis.*, 1913, 13, p. 294.

[3] *Jour. Infect. Dis.*, 1913, 12, p. 202.

Virus 2193 was diluted 1:15 in Ringer's solution and preparations made as before. Nine unincubated slides inoculated in the skin of a rabbit gave 6 flat pustules. Inoculation of the same number of preparations incubated 16 days (contaminated) produced 58 well-developed pustules.

FIG. 1.—Skin of rabbit showing on its right side no "take," only scars from shaving, following inoculation of five "immune" preparations; and on left side, a confluent "take" from inoculation of control preparations.

These two experiments show, then, that in spite of bacterial contamination, which may conceivably interfere with the growth of the specific microorganism, the virus was increased six- to tenfold by incubation in the cornea plasma preparations. In several other experiments in which a lower dilution of the virus was used, the extent of growth appeared to be greater, but in no

instance has the growth been comparable to that observed in cultures of rapidly growing bacteria. Irregularities in the rate of growth are to be expected, since they occur in the cultivation of practically all of the known microorganisms.

The method used here for estimating the degree of multiplication has been shown by Calmette and Guerin[1] to be reasonably accurate, comparing favorably with the method of plating for bacteria; it is in general use in commercial laboratories.

NECESSITY OF LIVING TISSUE FOR GROWTH OF VIRUS.

We have shown (first paper)[2] that when pieces of paraffin are substituted for cornea in plasma preparations, no growth of the virus takes place. To determine whether or not *living* cornea was necessary, the following experiments were carried out:

Experiment 503.—Virus 2186. Diluted 1:5 in Ringer's solution. The pieces of cornea to be used were divided into two lots; those in the first lot were frozen in an ice-salt mixture at −18° C. for 30 minutes, and were then used for virus cultures. The unfrozen tissue was used in control preparations. The frozen tissue upon incubation showed no outgrowth of either epithelium or connective tissue cells. Nine of these preparations incubated 12 days gave upon inoculation only 12–13 pustules; the same number of control preparations gave a confluent "take" composed of at least 300 pustules.

Experiment 504.—Virus 2186. Diluted 1:5. Pieces of cornea were placed for 45 minutes in weak hypotonic salt solution before being used. Inoculation of 9 unincubated preparations gave 50–60 pustules. Nine slides incubated 10 days were negative upon inoculation. There was also no "take" following the inoculation of 5 slides incubated 21 days. Microscopic examination showed that there was no growth of corneal tissue in any of the preparations. A second experiment was made at the same time using cornea killed by freezing as in Experiment 503. The results were practically identical with those just recorded for cornea killed by hypotonic salt solution.

These three experiments indicate that the vaccine virus does not grow in preparations containing corneal tissue killed by freezing or by a weak hypotonic salt solution. These two methods of destroying the vitality of the tissue were used because the chemical changes which they bring about are very slight. The results obtained are in harmony with the general conception of the close association of the virus with living body cells.

[1] *Ann. de l'Inst. Pasteur*, 1905, 19, p. 317.

[2] *Op. cit.*

SUBSTITUTION OF OTHER TISSUES FOR CORNEA.

Pieces of heart, liver, and kidney were used. Since the technic employed was the same as with cornea, it seems unnecessary to describe the experiments in detail. The results were as

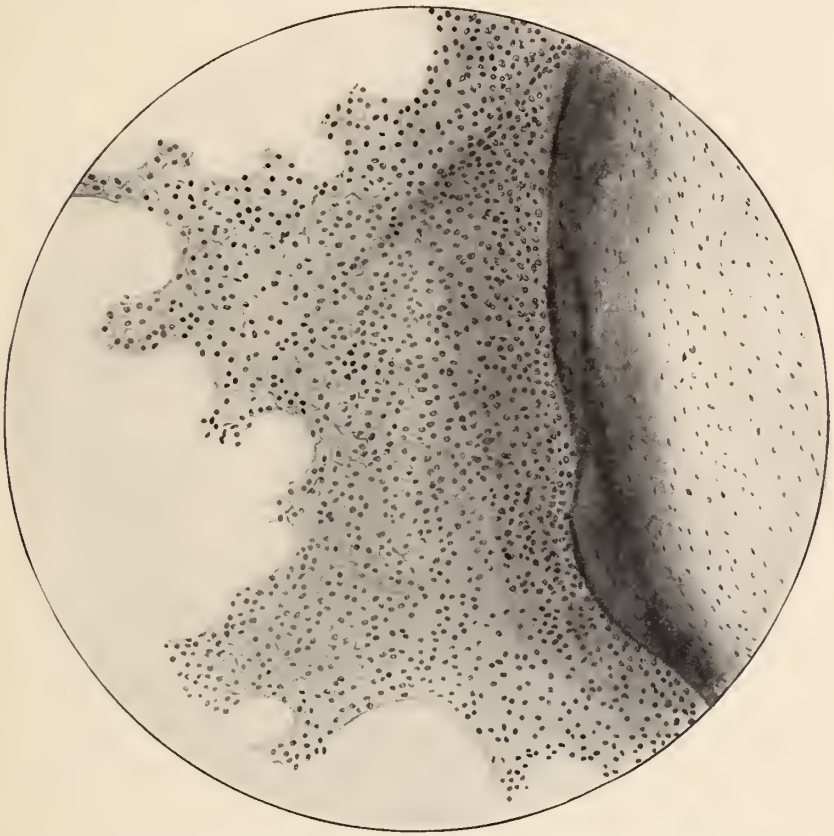

FIG. 2.—Tissue culture preparation of cornea showing outgrowth of a sheet of epithelial cells. Along the margin the sheet is only one cell thick, and the cell boundaries are quite distinct. The compact corneal fragment contains small oblong nuclei.

follows: The virus died out quickly in the liver preparations. This was due no doubt to the action of bile, which has been shown to be highly toxic for the virus. There was no evidence of multiplication in the heart and kidney preparations; on the contrary, there seemed to be a gradual weakening of the virus. Preparations

incubated for more than 10 days were found to be practically inactive.

USE OF PLASMA AND CORNEA FROM IMMUNE ANIMALS.

Immunity was established in a rabbit by a cutaneous inoculation of virus, resulting in an extensive eruption. Two weeks later the animal was bled, and the cornea removed for use in the following experiment.

Experiment 486.—Virus diluted 1:5. Two series of preparations were made: one, with plasma and cornea from an immune animal; the other, with plasma and cornea from a normal rabbit (controls). Inoculation of 5 "immune" preparations incubated 5 days gave a completely negative result (skin of rabbit, right side). Inoculation of same rabbit simultaneously on left side with an equal number of control preparations yielded a very extensive confluent "take." Repetition of this experiment a week later gave a similar result. In both experiments there were excellent growths of corneal epithelium.

This experiment shows that the virus becomes inactive in a short time in preparations containing plasma and cornea from an immune animal. The experiment also affords good proof of the adaptability of the method of tissue cultivation for the demonstration of many immunity reactions *in vitro*.

CONCLUSIONS.

1. The virus of vaccinia incubated in tissue cultures composed of plasma and cornea from normal rabbits and guinea-pigs shows a definite increase, but the degree of multiplication is not comparable to that observed in cultures of rapidly growing bacteria.

2. There is no growth of the virus in preparations containing cornea killed by freezing or by hypotonic salt solution.

3. There is no evidence of the growth of the virus in preparations in which pieces of heart, liver, or kidney have been substituted for cornea.

4. The virus is soon rendered inactive in preparations containing plasma and cornea obtained from an immune animal. The method of tissue cultivation is well adapted for the demonstration of immunity reactions *in vitro*.

THE SANITARY SIGNIFICANCE OF BODY CELLS IN MILK.*

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Cow's milk contains a certain number of cells derived from the tissues of the udder whose nature has been variously interpreted by different investigators. The presence of these tissue cells in colostral milk, where they have been spoken of as colostral corpuscles, has been recognized for a long time. The usual statement in textbooks and elsewhere is that these corpuscles are abundant for a few days after calving and then disappear. It has remained for the sanitarians of the last decade to show that tissue cells occur not only in colostral milk but in all milk.

Because of the occurrence of enormous fluctuations in numbers of these cells, it has been thought that these fluctuations have a sanitary significance. Many health officers who believe that large numbers of these cells indicate pathological conditions have condemned and still condemn milk on this basis. Thus the problem of interpreting the real significance of their presence becomes an important one.

The first person to direct attention to the presence of the cells in ordinary milk was Dr. Stokes of Baltimore, who published the first article discussing their sanitary significance in 1897.[1] He looked at the problem presented by their presence from the standpoint of the medical man and health officer, and, because he recognized some of them as white blood corpuscles, he naturally thought of them as "pus" cells, as these cells occur in great abundance in inflamed regions and in pus. Unfortunately, too many of the later studies of the cells in milk have been made from the same standpoint, a standpoint which has its limitations as well as its advantages. The particular limitation has been the tendency of other health officers to accept Dr. Stokes's interpretation of these cells as "pus" cells without question, and to talk about the presence of "pus" in the milk. Dr. Stokes tells me that he, himself, now regards this interpretation of their nature as unfortunate. Moreover, health officers are usually concerned with the examination of market milk samples, and are rarely in a position to trace abnormal conditions in any satisfactory way to their source. In many examinations of market milk abnormally large numbers of these cells have been found.

* Received for publication November 22, 1913.

[1] *Med. News*, 1897, 71, p. 45.

Frequently the same sample has shown large numbers of long chain streptococci of a type generally regarded as pathogenic. It has been assumed that the two were associated as cause and effect, the streptococci coming from pathological udders which were discharging large numbers of "pus" cells because of the presence of the streptococci. This assumption is possibly correct but rests on very insufficient data. Investigators have frequently traced their samples back to the herds from which the milk was obtained and, finding there a cow with garget, or a three-teated cow, or a fresh cow whose milk had been saved to the end of her lactation period, or suckling calves, or a poor, scrawny cow, have assumed, without tests, that these animals were responsible for the increased cell counts which they found. Inasmuch as it is uncommon to find a herd which does not have such animals, it is not surprising that they should have been found in practically all of these cases.

Moreover, almost all of the determinations of the numbers of cells present have been made by methods which were modifications of the first one used by Dr. Stokes; that is, the cells were counted in the sediment obtained by centrifuging, either by making stained dried smears from the sediment, or by counting the cells in the sediment by means of the Thoma-Zeiss blood cell counter.

Inasmuch as Professor Prescott and myself[1] showed some three years ago that centrifuge slimes sometimes contained as many as one-half of the cells and sometimes not more than one-fortieth of them even when the milk was centrifuged under the same conditions, one can readily see why the cell counts obtained in this way are not now regarded as accurate. Other investigations have shown that practically all of the cells rise with the cream in the case of gravity-raised cream and that the number of cells precipitated by centrifuges or separators is influenced by preliminary heating of the milk,[2] the speed of revolution, and probably other factors which make it impossible to secure consistent results even with the same centrifuge or separator.[3]

The following method has been used in all of the work referred to here: a small, measured drop of milk was taken directly without centrifuging and smeared over a known area on a glass slide, dried, stained, and the cells counted with a microscope. In this way the chance of throwing away from 50 per cent to 97.5 per cent of the cells has been eliminated. This method has its limitations and it is impossible to get duplicate tests to check absolutely, but in the hands of a reasonably careful person, there is no difficulty in getting duplicate cell counts to agree much better than duplicate plate counts for bacteria. There is no question but that the results secured represent a close approximation to the truth.

NATURE AND ORIGIN OF THE CELLS.

The first problem to be solved in regard to these cells is their nature and source. Three views have been held. They have been regarded (1) as leukocytes or white blood corpuscles, and because these cells occur in large numbers in inflamed regions and in "pus," they have frequently been thought of as "pus" cells;

[1] *Jour. Infect. Dis.*, 1910, 7, p. 632.

[2] *24th Ann. Rept., Wis. Agric. Exper. Sta.*, 1907, p. 231

[3] *Arch. f. Hyg.*, 1911, 75, p. 383.

(2) as epithelial cells and fragments of such cells derived from the epithelial lining of the secreting portion of the udder; (3) as a mixture of both kinds of cells.

All investigators admit that both white and red blood corpuscles occur under unusual conditions when blood makes its way directly into the milk.

In my opinion there is no question but that the third view is correct for the following reasons:

1. Because of the appearance of the cells. Certain of these cells are exactly like polymorphonuclear and polynuclear leukocytes. This type of cell has a nucleus of an unmistakable kind. No other cell in the body has one like it. These cells likewise stain characteristically in blood stains. Others of the cells closely resemble epithelial cells and may even occur in groups exactly as if they had clung together in being discharged from the lining of the alveolus. Nuclei and fragments of cells are also found which apparently belong to these epithelial cells.

2. Histologists recognize that it is a perfectly normal thing for both types of these cells to occur in the secretions of glands. For example, such cells frequently and almost constantly appear in saliva and in urine. White blood corpuscles normally make their way out through the walls of the capillaries into the connective tissues, and frequently make their way into the epithelial lining of the intestine, trachea, and other parts of the body. It is also normal to have epithelial cells discharged from epithelial surfaces, e.g., the lining of the mouth, and especially so in an active secreting surface such as is found in the alveoli of the udder. The secretion of the sebaceous glands is entirely composed of modified epithelial cells.

3. There is nothing in the appearance of histological preparations of the udder to justify the unusual interpretations which have been put upon the nature of these cells. When one studies preparations from udders which are known to have been actively discharging these cells at the time the animal was killed, there is no difficulty in finding white blood corpuscles abundantly in the connective tissues close about the alveoli. Similar cells, which stain in the same characteristic fashion, are found in the secretion

in the interior of the alveoli. Rarely, cells of the same type are found imbedded in the epithelium of the alveolus where they would be found if killed in the act of penetrating the lining of the alveolus. Free nuclei and entire cells of the same appearance as those found in the epithelial lining of the alveolus are also occasionally found in the interior of the alveoli, as they would be if detached from the secretory lining of the alveolus.

There is a continuous flow of large quantities of fluid from the lymph and blood vessels of the udder through the epithelial lining into the lumen of the alveolus in the process of milk secretion, and it is not at all surprising that the actively ameboid white blood corpuscles make their way through this epithelium with this current of fluid.

NORMAL NUMBER OF THESE CELLS FOUND IN MILK.

Before attempting to explain the significance of the presence of these cells it is necessary to know not only the nature and origin of the cells, but also the normal numbers and variations in numbers which occur.

The results obtained in counting the cells by the use of the method mentioned above have been much higher than those obtained by the examination of centrifuge slimes. At this time it is not possible to give the details of the examinations which have been made, but a brief outline may be given.

These examinations were first begun in Boston in 1910.[1] At this time 46 samples of milk from as many different herds in the neighborhood of Boston were found to contain an average of 1,485,000 cells per c.c. Eight market milk samples contained an average of 2,850,000 cells per c.c.

The following year[2] one of my students at Allegheny College, continuing the work, examined the milk of 37 cows from herds near Meadville, Pennsylvania, and found an average of 1,165,000 cells per c.c. He found great variation in the number of cells in the milk from different quarters of the udder. The largest number of cells occurred in the strippings. There was no constant relationship between the number of cells in the fore milk and that drawn during the latter part of the milking. This work was continued by the author at Göttingen, Germany, where the milk of 3 normal cows was examined daily for a period of 6 weeks. The daily variations in numbers of cells were found to be very large. One of these cows which was apparently normal in all respects was found to have very high cell counts; the highest one noted was

[1] *Jour. Infect. Dis.*, 1910, 7, p. 632.

[2] *Ibid.*, 1911, 8, p. 361.

5,975,000 per c.c. Individual quarters of the udder were tested on the same day and one quarter was found to be largely responsible for the high cell count. The milk from this quarter contained 22,500,000 cells per c.c., and yet this milk showed no evidence of being abnormal either in chemical analysis or in appearance. The cow was not suffering from garget, nor was a veterinarian able to find anything abnormal in her udder.

Numerous tests have been made of the herd at the experiment station at Geneva, New York. The average number of cells found in the milk of the 25 cows in the herd is 439,000 cells per c.c. Six cows have been studied in detail for periods of several weeks each. Two of these cows were fresh cows, 2 were abnormal because of abortion or troubles with the udder, and 2 were normal. A single examination has been made of the milk of each of 53 Guernsey cattle. The milk of these cows showed an average of 895,000 cells per c.c.

The data are presented in Table 1.

TABLE 1.

No. of Cows in Herd	Location	Average Number of Cells per c.c.	Number of Cows Giving Counts			Breed of Cow
			Between 0 and 500,000	Between 500,000 and 1,000,000	1,000,000 and up	
37.....	Meadville	1,165,000	10	12	15	Jersey and mixed grades
3.....	Germany	932,000	1	1	1	Harz and Glaner
25.....	Exper. Station	439,000	18	6	1	Jersey and grade Jersey
53.....	Geneva	895,000	27	16	10	Guernsey
118.....			56	35	27	Totals
Average 884,000 cells per c.c.						

There is no evident explanation of the striking differences between these herds. They may be chance differences or they may have some significance. Evidently it is not safe to generalize without more complete data.

It would be interesting to analyze these data with a view to discovering whether the 27 cows in these herds whose milk showed counts above 1,000,000 per c.c. had any characteristics in common whereby they differed from the animals whose milk contained fewer cells. Unfortunately, however, the records at hand do not justify any such discussion. The work thus far done is only in the nature of a preliminary survey and the histories of these animals are not known as completely as is necessary for such a discussion.

CONCLUSIONS.

From studies thus far made, certain conclusions may be drawn, some of which may be regarded as established while others are only tentative and are drawn up at this time merely in order to summarize our information to date.

1. Normal milk contains cells derived from the body of the cow which are of two entirely different types: (*a*) white blood corpuscles which are largely of the polynuclear and polymorphonuclear type; these cells make their way into the milk by passage through the epithelial lining of the secreting portion of the gland, possibly, also, through the epithelial lining of the ducts of the gland; (*b*) epithelial cells, nuclei, and cell débris discharged from the epithelial lining of the secreting portion of the gland and possibly also from the ducts.

2. The number of these cells in apparently normal milk is exceedingly variable even in the milk from the same cow. The variation in the number of cells in the milk from the different quadrants of the udder is almost as great as the variation in number of cells in the milk of different animals. Apparently the strippings always contain a greater number of cells than the milk from the earlier part of the milking.

3. It is very common to find milk that contains so few cells that they can scarcely be counted with the method of examination used, i.e., less than 5,000 per c.c., but milk containing one or more millions of these cells per c.c. is met with frequently. The highest cell count which has been found in this work was in the case of the strippings from one quarter of the udder of a cow 8 days after calving where the milk showed the enormous count of 54,300,000 cells per c.c. Nevertheless, this milk was of an entirely normal appearance and careful bacteriological examination of the udder showed no evidence of streptococcic infection. This milk had an entirely normal taste and caused no evil after-effects.

4. Out of 122 individual cows whose milk has been examined, 59 have been found to give cell counts under 500,000 per c.c., 36 gave counts between 500,000 and 1,000,000 per c.c., and 27 gave cell counts over 1,000,000 per c.c. The milk of all of these cows was normal in appearance and was sold or used by their owners, who had every reason to suppose that the milk was normal milk.

5. There are no satisfactory data at hand which show whether there is, or is not, a relation between high cell counts and any of the following: streptococcic infection of the udder or other patho-

logical conditions of the udder, colostral milk, milk from cows that are nearly dry, in heat, or in poor condition of flesh, etc. Such evidence as we have indicates that it is not at all likely that any of these conditions may be recognized by cell counts alone, especially in samples of market milk which consist of a mixture of the milk from several cows.

To answer the question implied in the title of this paper, "The Sanitary Significance of the Body Cells in Milk": it is entirely possible that some of the striking variations in numbers have a sanitary significance, as pathological conditions would certainly affect the discharge of these body cells. It seems probable, however, that the change produced by pathological conditions may not always be an increase in numbers of cells. Pathological disturbances in the blood are indicated just as surely by a decrease in numbers of red or white cells as by the reverse condition. Inasmuch as we have but very few data as yet on which to base conclusions, it is therefore impossible to make even a guess as to the final conclusions regarding the significance of the variations in number and character of the cells. The cells certainly do not have the significance of pus cells under ordinary conditions nor does it seem probable that it will be possible to recognize the admixture of pathological with normal milk by means of these cells alone. With our present knowledge it is certainly impossible to make this distinction.

THE PREPARATION OF DRIED CULTURES.*†

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INTRODUCTION.

The use of cultures in the dairy industry is of ancient origin, altho it is only in very recent years that the real nature of the cultures has been understood. While specially prepared cultures are now used in many creameries and even in therapeutics, a faulty technic has prevented the production of cultures of the highest purity and activity and has retarded their commercial development. All of the better class of creameries use pure cultures which are obtained at regular intervals from commercial laboratories. These are carried in the creameries by transferring milk cultures from day to day, the culture being renewed occasionally to insure its purity. The small milk culture known as a "mother starter" is usually carried in bottles or small jars, from which it is transferred to a large can or vat of milk to make the starter used to ripen the cream.

The ideal culture for distribution is in a dry form, sufficiently active to produce rapid growth when it is added to milk and yet so dormant that it can be held a long time without losing its activity. But the difficulties of producing a culture in this condition are so great that the most successful of the commercial cultures are distributed in a liquid medium and must be used within a comparatively short time. A few cultures, however, have been sold continuously in a powder or tablet form; in other cases the liquid culture has been found to be more satisfactory and the dry culture has been abandoned.

Recently the general interest in fermented milks, especially those of the yogurt type, has stimulated the production of various tablets and capsules which ostensibly contain the organism in such quantities that the culture can be used to start a fermentation in milk or to inoculate the digestive tract by direct consumption.

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† Published by permission of the Secretary of Agriculture.

These cultures, as well as those sold for butter-making and cheese-making, are of real value only when they contain a suitable organism free from contamination and are sufficiently active to start the acid fermentation before accidental contamination can develop to an appreciable extent.

CONDITION OF COMMERCIAL DRY CULTURES.

The poor quality of the ordinary yogurt tablet has been discussed in a previous paper.[1] Very few of the cultures examined in this laboratory have contained enough *B. bulgaricus* to sour milk before a large development of contaminating bacteria had occurred, and in many cases the contamination was so great that it was difficult to find the acid-forming organism at all. The butter cultures sold in the dry form are powders or tablets sometimes prepared with a filler of starch or lactose, or are milk cultures dried and pulverized.

TABLE 1.

SHOWING CONDITION OF COMMERCIAL CULTURES.

Culture	Bacteria per Gram	Contamination
A1	69,300	12 per cent
A2	492,700	Slight
A3	17,400	"
A4	6,520,000	"
B1	770,000	.....
B2	89,500	42 per cent
B3	365,000	Very high

The condition of various samples of cultures is shown in Table 1. One of these cultures, *A*, was a tablet, while *B* was evidently a dried-milk culture.

In preparing starters in accordance with the directions which accompany these cultures, a considerable portion of the powder is added to pasteurized or boiled milk. When this has curdled a small portion is transferred to fresh milk, and the process is repeated until a curd of good flavor and free from evidences of contamination is obtained. The result of this manipulation is the elimination of the contamination, usually very evident in the first transfer.

The rate of development of dried cultures when added to sterile milk and incubated at 30° C. is shown in Table 2. Culture *A* was

[1] *Circular 171*, U.S. Dept. of Agric., Bureau of Animal Industry, Washington, 1911.

a tablet, *B* and *C* were powders, while *D* was not a commercial culture but a powder prepared at our request by a special process which will be considered later.

In the commercial cultures examined nearly all of the bacteria had been destroyed in the preparation, and in one case the culture was almost sterile. It is obvious that in the ordinary process of drying cultures there is a great decrease in the number of bacteria originally present in the culture, and it is probable that there is a still further loss as the culture is held after drying.

TABLE 2.

CULTURES MADE FROM COMMERCIAL POWDERS.

CULTURE	RATIO OF POWDER TO MILK	BACTERIA PER GRAM OF POWDER	MILK AFTER 8 HRS. AT 30° C.		MILK AFTER 19 HRS. AT 30° C.	
			Acidity	Bacteria per c.c.	Acidity	Bacteria per c.c.
			Percentage		Percentage	
A	1:200	0	.216	0	.261	0
B	1:200	.....	.261	103,500	.855	1,275,000,000
C	1:200	.....	.225	101,500	.819	90,500,000
D	1:200	6,120,000,000	.819	1,895,000,000	1.071	1,280,000,000
A	1:1000	.....	.270	12,000	.279	9,300,000
B	1:1000	3,650,000	.261	1,150,000	.684	1,705,000,000
C	1:1000	.....	.252	2,000	.270	34,500,000
D	1:1000	8,590,000,000	.387	223,000,000	.855	1,840,000,000
A	1:500	69,300	.207	47,750,000	.414	322,000,000
B	1:500	89,500	.203	2,230,000	.819	635,000,000
C	1:500	.....	.198	15,000	.684	135,500,000
D	1:500	2,015,000,000	.342	526,500,000	.999	1,710,000,000

DECREASE OF BACTERIA IN DRYING.

A freshly curdled milk culture of the ordinary lactic acid bacteria usually contains about 1,000,000,000 bacteria per c.c. If this were reduced to a water-free powder each gram would contain approximately 100,000,000,000 bacteria. The usual process of drying cultures consists in reducing the percentage of water by adding a dry substance, as for instance, powdered lactose, and exposing the mixture to a current of air at a temperature sufficiently high to cause a rapid evaporation. In order to insure a reasonably rapid loss of water it is necessary to hold the culture at a temperature somewhat above the optimum, and, with some cultures, above the upper temperature limit of growth. As the drying progresses, there is a concentration of the acid which there is every reason to believe would

have a detrimental effect on the bacteria. There is also a concentration of other solids in solution in the water of the culture, and there must come a point when the concentration of the solids in the water surrounding the cells becomes so great that a large part of the water is withdrawn by the osmotic pressure and plasmolysis results.

Table 3 shows the moisture and bacterial content of a culture at half-hour periods during the drying. The apparatus in which this preparation was made consisted of a water oven, through which was blown air previously dried by passing over pumice stone saturated with sulfuric acid and warmed in a coil of lead tubing submerged in a water bath. The oven was maintained at a temperature of 40° to 43° C. The current of warm dry air was directed against the moist culture so that the evaporation was as great as possible. The culture used in this experiment was 40 gms. of a freshly curdled milk culture of a typical lactic organism to which were added 20 gms. of sterile pulverized lactose.

TABLE 3.

RELATION OF LOSS OF MOISTURE TO DECREASE IN BACTERIA.

MINUTES FROM BEGINNING OF DRYING	MOISTURE	BACTERIA PER GRAM	
		Moist Powder	Water-Free Basis
	Percentage		
0	59.05	785,000,000	1,917,000,000
30	48.05	750,000,000	1,443,000,000
60	34.71	963,000,000	1,475,000,000
90	24.05	942,000,000	1,240,000,000
120	10.56	916,000,000	1,024,000,000
150	4.74	351,000,000	368,000,000
1020	3.25	385,000,000	393,000,000

In the first 2 hours there was a gradual decrease in the bacteria of nearly 50 per cent, with a drop in the water content to 10.56 per cent. This decrease was fairly uniform in the different periods and may be attributed to the unfavorable temperature conditions to which the culture was exposed.

In the next period of 30 minutes, in which the water content changed from 10.56 per cent to 4.74 per cent, the bacteria dropped from 1,024,000,000 to 368,000,000 per gram. At this time the culture was removed from the drying oven and held until the next morning in a desiccator at room temperature. In this period there was no further decrease in bacteria.

It is evident from this experiment, which was supported by others of a similar nature, that at a water content of between 5 and 10 per cent a concentration is reached which is fatal to a large number of cells. Those that survive become dormant and remain alive for an indefinite period.

LOSS IN RAPID DRYING.

If the assumption be true that exposure to a concentrated medium is responsible in large measure for the death of the bacteria, a method of drying which reduces the time of exposure to this unfavorable condition should diminish the loss of bacteria in the drying process. Fortunately we were able to have a number of cultures dried by a process in which the water was removed from the milk by a spray carried up by a current of warm, dry air. The drying was completed in a very short time, and on account of the rapid evaporation, took place at a low temperature. The cultures dried were of an active lactic acid organism grown on milk. The number of bacteria per gram in various powders made by this method is given in Table 4.

TABLE 4.

BACTERIA IN CULTURES DRIED BY SPRAYING.

Powder No.	Bacteria per Gram	Powder No.	Bacteria per Gram
1	748,500,000	7	8,590,000,000
2	1,933,500,000	8	5,845,000,000
3	4,010,000,000	9	1,775,000,000
4	6,120,000,000	10	4,862,000,000
5	1,490,000,000	11	657,500,000
6	2,015,000,000		

These results show that while there is still a large decrease from the theoretical number present in the milk, the rapid drying gave a powder with a very high bacterial content. These powders gave an active growth when added to milk and produced a good starter on the first inoculation. However, the manufacture of a dry culture by this process requires the use of complicated and expensive machinery and is therefore not within the reach of the ordinary laboratory.

DRYING BY THE FREEZING METHOD.

It is well known that water vapor is given off from ice even when the temperature of the air is below the freezing point of water. If the atmospheric pressure is lowered the rate of evaporation is increased, and if the vapor is removed as fast as it is formed so that the vapor tension is not too high, the entire piece of ice may be evaporated without passing through the fluid state.

This principle has been utilized in a laboratory method of drying first described by Shackell.[1] The method used by him consisted essentially in placing the frozen material in a desiccator over sulfuric acid and exhausting the air either by adding a small amount of ethyl ether and exhausting with a water pump or exhausting the air with a Geryk pump without the aid of ether. The ether vaporizes, displacing the last traces of air, and is itself absorbed by the sulfuric acid. The drying was hastened by rotating the desiccator to replace the saturated layer on the surface with fresh acid. Schackell used this chiefly for moisture determinations but also pointed out its application to other biological problems. Blood was dried without the loss of the dissolved gases or property of clotting and the brain of a rabbit affected with rabies was dried without losing its virulence. Shackell points out that this is probably due to the fact that there is no concentration of the soluble constituents during the drying. The concentration usually incident to drying is avoided because there is no fluid water to hold the solids in solution as the drying progresses.

If the process is interrupted before the drying is complete, it will be found that the outer part of the material will be completely dry, while the inner part is in its original condition. The dry part is porous and friable. Substances which cannot be dried by other means without becoming insoluble in water may by this method be reduced to a powder which is readily soluble in water. The possible value of this method in preparing dry cultures is obvious. Not only is the injurious effect of the increasing concentration during drying removed, but the process is conducted with the cells in a dormant condition.

Hammer[2] compared this method with air drying by moistening strips of filter paper in broth cultures and holding over sulfuric acid in a desiccator submerged in salt and ice and evacuated by a water pump. Cultures dried in this way showed a much greater viability than similar cultures dried over sulfuric acid in an unevacuated desiccator.

Shattock[3] and Dudgeon dried cultures on slips of glass. With some organisms there was little or no difference in the viability of the cultures dried in a vacuum after freezing and those dried in air, but with others, notably *B. pyocyaneus*, the difference in favor of the freezing method was very marked.

The success of this method necessarily depends on the ability of the organism to withstand freezing. It is generally known that many bacteria will survive for long periods in ice, but it is possible that the freezing would destroy a sufficiently large proportion of the bacteria in a culture to affect the activity of the powder.

Macfadyen[4] and Rowland found that *B. acidi lactici*, *B. typhosus*, *B. coli communis*, and many other bacteria as well as a yeast culture survived 10 hours' exposure to a temperature of −252° C. without change in appearance or vitality.

Paul and Prall,[5] who dried staphylococci on garnets and exposed them for one month to the temperature of liquid air, found no diminution in numbers in this period and no change in their resistance to disinfectants. Smith and Swingle[6] found, on the other hand, that when broth cultures are frozen and thawed a large number of the cells succumb and only certain more resistant cells survive. It is possible that the process of concentration taking place as the fluid freezes may have some influence on the

[1] *Am. Jour. Physiol.*, 1909, 24, p. 325.
[2] *Jour. Med. Research*, 1911, N.S., 19, p. 527.
[3] *Proc. Royal Soc.*, 1912, 85, p. 127.
[4] *Ibid.*, 1900, 66, p. 488.
[5] *Arb. a. d. k. Gsndhtsamte.*, 1907, 26, p. 73.
[6] *Science*, 1905, N.S., 21, p. 481.

vitality of the cells. The few determinations that we have made with milk cultures have given no indication of any serious loss due to the freezing. This is seen in Table 5, which shows the effect of freezing on cultures of the lactic bacteria.

These results are substantiated by the activity of the dry cultures made from frozen milk. It is evident that a sufficiently large number, at least, survive the freezing to permit the production of a very active powder.

TABLE 5.

EFFECT OF FREEZING ON BACTERIA IN MILK CULTURES.

POWDER NO.	BACTERIA PER C.C.	
	Before Freezing	After Freezing
1	2,305,000,000	2,290,000,000
2	2,125,000,000	1,920,000,000
3	890,000,000	1,110,000,000
4	2,300,000,000	1,455,000,000

METHOD.

In our preliminary work we have used a desiccator for a drying chamber which is very satisfactory for drying small quantities. The culture was usually frozen by flowing in a petri dish and submerging in a mixture of salt and ice. Material may be frozen more quickly and neatly by the use of carbon dioxid snow. We have obtained this snow in the following manner.[1] A one-fourth inch needle valve is connected to the carbon dioxid cylinder by a short piece of pipe, the cylinder inverted, and the valve opened so that the liquid flows into the pipe. This arrangement avoids both the loss due to the cooling of the head of the cylinder as the liquid expands and the plugging of the opening by the formation of ice. A bag made of three or four thicknesses of felt and held in shape by a large cork tacked in one end is held over the end of a pipe a few inches long leading from the needle valve; the valve is then opened, and the snow which forms as the liquid carbon dioxid expands is collected in the bag. More efficient results in freezing may be obtained if the snow is mixed with alcohol, gasoline, or a similar reagent, but for ordinary purposes it is necessary only to place the pan of material to be frozen on a layer of dry snow. The desiccator containing the frozen material was partly covered with salt and ice and the joint between the cover and the desiccator well lubricated with a mixture made by melting together gum rubber and vaseline. Sufficient vaseline must be used to make a soft mixture on the cold glass. When the desiccator is closed the cover should be rotated slightly to eliminate channels through the lubricant. The desiccator is connected with the house vacuum to remove as much air as possible and then with a Geryk pump to secure the high vacuum necessary for rapid drying. The air is passed from the desiccator through a drying train in which it passes in succession over calcium chlorid, sulfuric acid, and phosphorous pentoxid.

[1] The writer is indebted to Mr. F. S. Durston, of the Bureau of Standards, for this suggestion.

We have constructed a drying train from gas-washing towers which has been very satisfactory, not only from the standpoint of efficiency but also on account of the ease with which the drying agents may be replenished. The gas-washing towers are placed in a horizontal position as shown in Fig. 1, and connected with soft rubber tubing which is covered by a mercury seal. The ground joints are well lubricated and rotated carefully to break any channels. It is necessary to make rubber connections between the desiccator and the drying train and between the drying train and the pump. Heavy pressure tubing is used for this purpose. Connected with the drying train is a Plücker tube to indicate the state of the vacuum. It is absolutely essential to the process that a high vacuum be maintained, and to secure this the joints must be perfect and all moisture removed from the air before it passes into the pump.

When a vacuum sufficiently high to produce rapid drying has been secured, the Plücker tube will show a fluorescence indicating a vacuum of about 0.01 mm. The time required to produce this will depend, of course, on the efficiency of the pump and the drying train and the capacity of the desiccator. With our apparatus, using one desiccator, it usually required less than half an hour.

The time required for complete drying varies with the relative amount of material and sulfuric acid, the relative amount of surface exposed in the material, and the completeness of the vacuum. We usually dried in a desiccator about 10 c.c. of milk culture. Under ordinary circumstances this would be completely dried in 3 or 4 hours, but we have made a practice of disconnecting the desiccator when the necessary vacuum is secured and holding overnight in a refrigerator maintained at a temperature a few degrees below freezing.

COLLECTING THE MOISTURE.

It is very essential to the success of the process that an efficient agent be provided to absorb the moisture as rapidly as it is given off by the material undergoing desiccation. The difference in vapor tension between the material and the absorbent must be great in order to insure a rapid transfer of moisture from one to the other. Phosphorus pentoxid answers this requirement, but on account of its cost and the nature of this reagent which makes it disagreeable to handle it is not well adapted for use on an extensive scale. Concentrated sulfuric acid which has a vapor tension near zero is nearly as efficient and has the advanatge of being available for repeated use by reconcentrating. Lime, which has the advantage of cheapness and of presenting a very large surface, has not been found satisfactory. Its action is too slow to make it a possible substitute for sulfuric acid.

Dr. Frankland of the Public Health Service has suggested the possibility of condensing moisture in a secondary chamber held at a very low temperature and has successfully used liquid air for this

purpose. The vapor tension of ice at 0° C. is 4.579, while at −50° C., the lowest temperature given in Landolt-Börnstein's tables, the vapor tension is 0.034. At the temperature of liquid air, which is about −175° C., the vapor tension would be very low indeed, and there would be a rapid transfer of moisture from the ice at 0° to that at −175°. Carbon dioxid gas, which is the reagent used in many refrigerating machines, has a boiling point of −78° C. Ammonia, which is more commonly used in this country, gives, with a back pressure of 10 pounds, a temperature of about −35° C., which corresponds to a vapor tension of 0.173.

One experiment was made using carbon dioxid snow to cool a condenser connected with a desiccator. A small desiccator was connected by a one-half-inch tube with a round-bottomed flask of 500 c.c. capacity. The tube was cemented to the desiccator and the flask. In the desiccator was placed a crystallizing dish containing 10 gms. of ice. The desiccator was packed in ice mixed with a small amount of salt. When the system was evacuated to about 0.01 mm. the flask was packed in carbon dioxid snow and held in this way for 4 hours. At the end of this time it was found that the ice had lost only about 0.3 gm. While the difference in vapor tension between the ice in the desiccator and that on the inner surface of the flask which served as a condenser was great, it evidently was not sufficient to cause a rapid transfer of moisture from one to the other.

DRYING IN LARGE QUANTITIES.

In order to secure larger quantities of powder a large drying chamber was constructed, using a 10-inch cast-iron pipe with one end closed by a stock cap. On the top was threaded a standard cast-iron flange fitted with a heavy cast-iron cover. The cover and the flange were ground true and a tight joint made by a rubber gasket. It was necessary, however, to clamp the cover down to make a perfectly tight joint. Some difficulty was encountered at first on account of a pinhole through the cast-iron cover, but this was overcome by painting the cover with shellac.

The drying chamber was held in an insulated tank so constructed that salt and ice could be packed about it in sufficient quantities to hold the temperature below freezing for several hours. A rack supporting 10 circular shelves was used to hold 4 lead pans for sulfuric acid and 6 tin trays for the culture. The cultures were frozen in the trays by packing in salt and ice, the covers removed and the trays placed on the shelves, alternating with the pans of acid. The rack was placed in the drying chamber, the cover clamped in place, and water added to the ice and salt mixture in the tank until the joint between the cover and the flange was covered. It is very difficult to make a rubber-joint vacuum tight, but by covering the rubber with a fluid this can usually be accomplished. As much as possible of the air was then removed by a connection with the house vacuum and the residue exhausted with the Geryk pump. With our apparatus this usually required 3 or 4 hours.

To avoid the necessity of submerging the tank in salt and ice a new apparatus was constructed as shown diagrammatically in Fig. 1. In place of the tank the drying

chamber is held in a horizontal position in a refrigerator maintained at a temperature below freezing by brine coils. The flange is grooved to hold two gaskets as shown in Fig. 2, thus providing a space which can be filled by means of a rubber tube connected at the lower opening, *A*, with brine, oil, or mercury. The vent, *B*, permits the escape

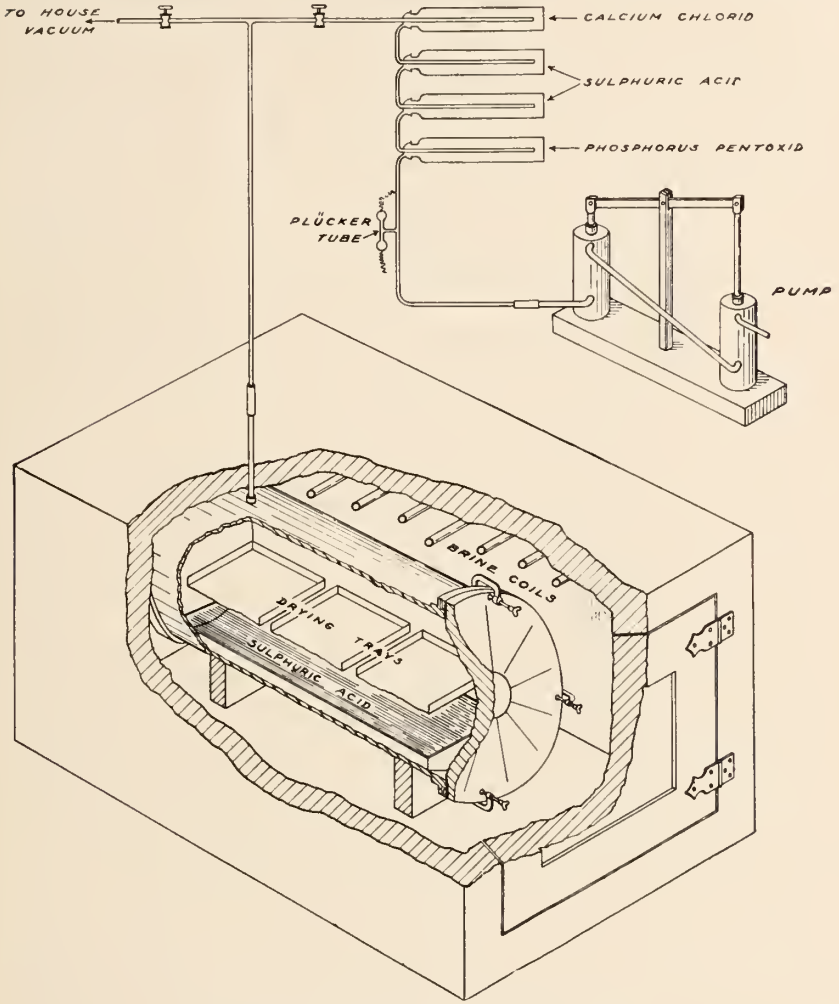

FIG. 1.—Apparatus for drying large quantities of culture.

of the air as the fluid fills the annular space between the gaskets. In this way a vacuum-tight joint may be obtained without excessive pressure on the cover. Similar results may be obtained by using a lead gasket, but this requires a high pressure on the cover to insure a tight joint.

Drying Lactic Cultures.

In drying by this process a freshly curdled milk culture was used. This may be ordinary sterile milk inoculated and incubated overnight at 30° C., or, for reasons which will be pointed out later, cultures may be made on milk concentrated to one-half to one-fourth of its original volume. The culture, which is frozen hard, comes from the drier in a very friable condition and is easily reduced to a powder. Powders made from concentrated milk contain so much sugar that they become slightly plastic shortly after they are taken from the drier. However, this does not prevent their being ground in a mortar.

Table 6 shows the number of bacteria per gram in various powders made from cultures suitable for cream ripening.

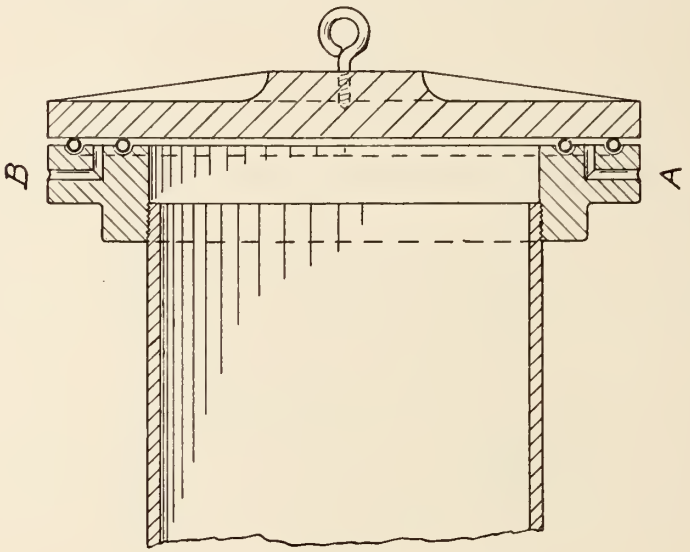

Fig. 2.—Arrangement for obtaining a vacuum-tight joint.

It is difficult to get an accurate count on these powders because the particles of acid curd are not readily soluble in the dilution water and undoubtedly many bacteria are held in these particles. Notwithstanding this fact Table 6 shows that a powder containing a large number of bacteria can be made by this process.

TABLE 6.

Bacteria in Lactic Cultures Dried by Freezing Method.

Powder No.	Bacteria per Gram	Powder No.	Bacteria per Gram
1	8,030,000,000	7	380,000,000
2	7,737,000,000	8	4,900,000,000
3	12,670,000,000	9	724,000,000
4	6,770,000,000	10	929,000,000
5	10,180,000,000	11	1,020,000,000
6	385,000,000		

The number of bacteria found by the plating method is not always a good indication of the activity of the powder as an agent for souring milk, and another method has been used to test this point. One milligram weighed on a chemical balance is added to 1,000 c.c. of sterile milk and incubated at 30° C. The activity of the powder is indicated by the increase in acidity of the milk, which is titrated at stated intervals. On account of the difficulty of weighing out this small amount of powder with even reasonable exactness the procedure was varied by weighing one-half a gram into a water blank from which a definite dilution may be made. The probable accuracy of this method is shown in Table 7, which gives the results obtained by making six separate inoculations from one lot of powder in 1000 c.c. flasks of milk.

In three of the flasks the powder was added in the ratio of 1 to 1,000,000 and in the others in a ratio of 1 to 40,000,000. It is evident that while this method gives a good indication of the activity of the powder, it can be depended upon for minor differences only when similar results are obtained on repeated trials.

The activity of a number of powders tested in this way may be seen in Table 9, on p. 113. The milks were inoculated with powder at the rate of 1 part of powder in 40,000,000 parts of milk.

TABLE 7.

METHOD OF TESTING CULTURES.

DILUTION OF POWDER IN MILK	ACIDITY OF MILK AFTER	
	18 Hrs.	20 Hrs.
	Percentage	Percentage
1:1,000,000	.594	.720
	.729	.783
	.684	.756
1:40,000,000	.207	.315
	.189	.261
	.198	.306

These results should be compared with those given in Table 2, which shows the acidity developed by commercial cultures under similar conditions except that the dilution used was very much less. The cultures dried by freezing gave a clean-flavored curd on the first inoculation without evidences of contamination.

INCREASING THE NUMBER OF BACTERIA BY NEUTRALIZATION.

In order to secure a powder with the required number of active bacteria it is necessary to start with a culture containing a large number of growing cells. A freshly curdled milk culture of an ordinary lactic bacterium will usually contain, by the accepted methods of counting, 1 to 3 billions per cubic centimeter. This will depend somewhat on the activity of the culture, and some strains may cease multiplication before this number is reached. It is well known that the growth period may be extended by various methods of neutralization and this has been made use of in the addition of phosphates to sugar broths. An attempt was made to increase the number of bacteria in milk cultures before drying by this means. Sterile milk containing 1 per cent dibasic potassium phosphate was inoculated with a freshly curdled

milk culture and held with suitable check without potassium phosphate at 30° C. The results are given in Table 8.

TABLE 8.

INFLUENCE OF POTASSIUM PHOSPHATE ON GROWTH OF BACTERIA.

Lot No.	Age	Acidity		Bacteria (Millions per c.c.)	
		Normal	Potassium Phosphate	Normal	Potassium Phosphate
	Hrs.	Percentage	Percentage		
1	0	.216	.297	7	8
	12	.801	1.059	2,590	536
	15	.882	1.332	3,640	5,020
	19	.918	1.449	2,690	4,830
	36	1.071	1.615	2,890	18,880
2	0	.....	.....	7	6
	12	.669	.779	2,630	1,400
	15	.801	.801	2,340	2,850
	19	.826	.829	2,290	2,100
	40	.855	.963	2,550	1,730
3	0	.243	.237	9	8
	12	.871	1.180	1,620	2,960
	15	.918	1.198	1,690	2,570
	19	.972	1.198	2,130	2,060
	36	.972	1.242	2,770	3,820
4	0	.297	.297	4	4
	12	.756	.855	2,530	3,160
	15	.880	1.282	2,210	4,150
	19	.900	1.395	.....	.....
	36	.999	1.638	2,510	4,340
5	0	.200	.255	8	8
	12	.715	.994	2,420	3,470
	15	.846	1.166	2,780	3,650
	18	.846	1.224	2,540	4,260
	36	.972	1.368	2,360	3,990
6	0	.155	.216	9	10
	2	.171	.243	111	93
	4	.186	.257	220	219
	6	.252	.576	650	700
	10	.720	.860	2,580	3,300
	24	.949	1.521	2,520	5,070
	30	.936	1.476	2,770	4,680
7	0	.167	.239	9	7
	2	.180	.254	14	10
	4	.196	.266	80	69
	7	.257	.326	743	169
	11	.703	.745	2,320	2,720
	24	.967	1.363	3,310	3,550
	31	.999	1.404	5,070	4,770
8	0	.167	.239	8	6
	2	.180	.248	6	15
	4	.194	.252	9	59
	7	.247	.279	230	327
	11	.531	.540	322	2,240
	24	.837	1.359	2,480	4,790
	31	.909	1.422	1,480	3,940

The number of bacteria in milk in which the acidity was neutralized by potassium phosphate was usually considerably greater than in the corresponding unneutralized culture, but when these milks were dried by the spraying method the unneutralized milks gave the more active powder. Evidently the acid phosphate had a detrimental effect when it was concentrated.

Somewhat similar results were obtained in cultures made by the freezing method from lactic milk cultures one-half of which were neutralized by the addition of 2 per cent calcium carbonate. The results, which are given in Table 9, are contradictory and show no special advantage in neutralizing before drying.

TABLE 9.

EFFECT OF NEUTRALIZING MILK BEFORE DRYING ON ACTIVITY OF CULTURE.

POWDER NO.	TIME FROM INOCULATION	ACIDITY OF MILK INOCULATED WITH CULTURE FROM	
		Unneutralized Milk	2 Per Cent $CaCO_3$
	Hrs.	Percentage	Percentage
1	17	.194	.347
	19	.288	.661
	21	.626	.779
2	17	.200	.270
	19	.297	.594
	21	.513	.675
3	17	.153	.153
	22	.243	.207
	24	.567	.360
4	17	.702	.702
	19	.837	.837
5	17	.414	.189
	23	.738	.468
	25	.738	.675
6	18	.738	.693
	20	.792	.738
7	17	.216	.247
	19	.342	.427
	21	.666	.693
8	17	.171	.185
	19	.211	.216
	21	.333	.302
	23	.598	.545
9	18	.288	.216
	20	.468	.378

By neutralizing *B. bulgaricus* cultures at different stages of growth somewhat similar results were obtained. Cultures in milk concentrated to one-half its volume were grown 24 hours at 37° C. At the end of that time a portion was dried and the remainder partially neutralized and again incubated. This could not be done accurately on account of the high percentage of casein. After 48 hours a second portion was dried and the remainder neutralized, and at 72 hours the third portion was dried. The three lots of powder thus obtained were tested by adding 10 milligrams to 500 c.c. of milk which was incubated at 37° C. and the acidity titrated at regular intervals. The results, which are given in Table 10, show that the most active powders are obtained from the unneutralized milk.

The neutralization evidently did not stimulate the growth of this organism. On the contrary the milk of Portion 5 contained at 24 hours 12,400,000 per cubic centimeter, at 48 hours 3,500,000 per cubic centimeter, and at 72 hours only 100,000 per cubic centimeter.

By Concentration of the Milk.

The observation was made by Mr. Ayres of this laboratory in the course of another investigation that *B. bulgaricus* gave a more vigorous growth in a concentrated milk or whey. It was thought that similar methods with the ordinary lactic type might produce a more active powder. To test this a quantity of milk, concentrated in a vacuum to one-half its volume and sterilized in an autoclav, was inoculated with a good lactic culture and incubated at 30° C. for about 18 hours. Similar cultures were made in normal sterile milk and incubated with the concentrated-milk culture. When these were dried by the freezing method and compared by inoculating a definite amount into milk, as shown in Table 11, there was no great difference in the activity of the two powders.

TABLE 10.

Influence of Neutralization on Activity of Bulgaricus Powders.

Powder No.	24-Hour Culture Not Neutralized				48-Hour Culture Neutralized at 24 Hours				72-Hour Culture Neutralized at 24 and 48 Hours			
	Acidity of Milk Inoculated with Powder after				Acidity of Milk Inoculated with Powder after				Acidity of Milk Inoculated with Powder after			
	17 Hrs.	19 Hrs.	21 Hrs.	23 Hrs.	17 Hrs.	19 Hrs.	22 Hrs.	23 Hrs.	17 Hrs.	19 Hrs.	21 Hrs.	23 Hrs.
	%	%	%	%	%	%	%	%	%	%	%	%
1	.261	.396	.594	.738	.177	.180	.207	.216	.....	.....	.....	.....
2	.279	.333	.387	.621	.135	.144	.153	.171	.144	.153	.153	.162
3	.693	.711	.720	.738	.232	.288	.387	.576	.189	.261	.342	.423
4	.180	.180	.198	.243	.162	.198	.288	.630	.153	.180	.243	.441
5	.....	.450	.522	.666	.....	.333	.432	.603	.....	.279	.297	.360

TABLE 11.

Relative Activity of Powders from Plain and Concentrated Milk.

Powder No.	Dilution of Powder	Powder from Plain Milk			Powder from Concentrated Milk		
		17 Hrs.	19 Hrs.	21 Hrs.	17 Hrs.	19 Hrs.	21 Hrs.
		Percentage	Percentage	Percentage	Percentage	Percentage	Percentage
1	1: 1,000,000	.702	.837	.....	.531	.774	.....
2	1: 1,000,000	.738	.792	.....	.666	.738	.....
3	1:40,000,000	.216	.342	.666	.207	.333	.616

There is, however, a decided advantage in the use of the concentrated milk. Weight for weight, the powder is as active as that obtained by drying plain milk, and since about half of the water has been removed before drying, twice as much powder can be produced in each operation of the drier when a culture grown on concentrated milk is used. For laboratory purposes concentrated milk can be easily produced by boiling skim milk in a large round-bottomed flask connected by a condenser with a large vacuum flask.

If a vacuum of 26 inches or more is maintained, rapid evaporation takes place at a low temperature. Milk may be concentrated in this way to one-fourth its original volume, but on sterilizing this forms a thick coagulum that is difficult to handle, so that milk concentrated to one-half its volume is more satisfactory.

DRYING *B. Bulgaricus*.

The great demand for cultures of this group to use in fermenting milk or for direct consumption for therapeutic purposes has given special interest to attempts to produce an active powder by the freezing method. The commercial powders made in various ways have not proved satisfactory, due in part to the crude methods of drying and partly to the fact that the decrease in activity after drying is rapid.

Our results have shown that it is possible to make a dry culture of *B. bulgaricus* containing over 1,000,000,000 cells per gm. These powders, made by the freezing method, when added to milk in the ratio of 1 part of powder to 1,000,000 parts of milk and incubated at 37° C., give an acidity of from 0.6 to 0.8 per cent in 24 hours and a curd free from any evidences of contamination. The best powder is made from a culture grown on concentrated milk 48 hours at 37° C.

In using these organisms for therapeutic purposes there are certain advantages in an active powder, in addition to its use in preparing sour milk. The culture is used to introduce into the digestive tract as many acid-forming organisms as possible, and for this purpose probably nothing is better than a good milk culture. But in many cases the patient objects to the taste of the milk, or it is inadvisable to introduce into the diet so much nitrogenous material. There is also the difficulty of obtaining or preparing the milk culture. Under these conditions a dry powder containing a large number of active cells would be of great advantage.

DRYING LABORATORY CULTURES.

The difficulties in carrying stock cultures are too well recognized to need discussion. Frequent transfers are necessary to insure the maintenance of many cultures, there is always the danger of contamination by molds, and the salient characters of some cultures are slowly changed by continued growth on artificial media. Every bacteriologist has been confronted with the question of discarding cultures which may at some indefinite future time be of great value or of continuing their transfer at considerable trouble and with the knowledge that many of them will eventually be lost. The advantage of having a culture in such a condition that it can be put away in the refrigerator and held for months or years with its characters unimpaired is obvious.

We have dried a large number of cultures of various kinds, and altho the time since drying has not yet been long enough to warrant definite conclusions, it is evident that the cultures may be maintained in the dry condition for many months, especially if they are held in a refrigerator. These cultures were dried at first in small phials plugged with cotton. It was found, however, that ordinary test tubes were more satisfactory for this purpose. The tube should be of a large diameter, and the plug should be loose to permit a free flow of air and moisture out of the tube. If too much of the medium is used it will not become dry in a reasonable time. We have found that when drying milk cultures 3 or 4 c.c. is sufficient. This dries quickly and gives an abundance of powder.

We have dried in this way many lactic-acid cultures, cultures occurring in Swiss cheese, and cultures of the *coli* group. Much trouble has been experienced from the difficulty in keeping cultures of the latter group in a frozen condition, due, no doubt, to the amount of alcohol and other volatile products formed by the fermentation of the sugar. This difficulty has been obviated by growing them in a medium made by adding 2 or 3 per cent of casein to sugar-free broth. Sufficient growth is obtained without the formation of alcohol and the casein acts as a filler for the powder.

It is apparent, however, that the ability to withstand this process varies with different organisms. It is also possible that varying results may be due to the inability of some organisms to give a luxuriant growth in the medium used. Whatever the cause may be, the number of failures to revive after long periods has been great enough to preclude an unconditional recommendation for this purpose.

Drying Cultures of the Legume Bacteria.

Within recent years the advantage of inoculating certain soils has been abundantly demonstrated, and various methods have been devised for distributing cultures for this purpose. None of the dried cultures have proved to be entirely satisfactory, and the legume bacteria are disseminated by fluid or semi-fluid cultures or by the transfer of infected soil.

A culture of this type isolated from the nodules of sweet clover and grown in milk was dried by the freezing method and a powder obtained containing in each gram about 1,000,000,000 cells. After 6 months at room temperature the powder gave typical growth in a dilution of 1 to 1,000,000, but no growth in the 1 to 10,000,000 dilution.

Since this organism does not ferment lactose, no appreciable acidity is formed in the milk and the powder obtained dissolves quickly and completely in water.

Drying Yeasts.

It is probable that yeast cells are too large to be dried by any method requiring freezing. Cultures of a bread-yeast fermenting saccharose but not lactose have been dried, but the powder when reinoculated into milk containing saccharose gave evidence of gas formation only after several days' incubation. Similar results were obtained with a powder made by freezing and drying the sediment obtained by centrifuging a dextrose broth culture of the bread yeast. The powder obtained in this way was composed almost entirely of yeast cells which were, so far as microscopic examinations showed, intact, but gave no fermentation when inoculated into sugar broth.

CONDITIONS INFLUENCING LOSS OF VITALITY.

The question of retention of vitality is of scientific as well as practical importance. Any culture gradually loses its vitality until it can no longer be reproduced. The rate of this loss is dependent on several conditions, all of which have an influence on the functions which maintain the vital activities of the organism. A culture grows more rapidly, reproduces itself more quickly, and dies earlier at its optimum temperature than at temperatures near the lower thermal limits of its activity. The same relations hold at temperatures too low to permit actual growth. An organism whose normal life cycle occupies a few days may live for months in a dormant condition, but even this existence is limited. A seed that will germinate in a few days in a moist condition may retain the power of

germination for months or even years if the moisture is withheld. The nearer the cell approaches an absolutely dormant condition the longer its actual death will be postponed.

The dormancy is necessarily only relative, as it is hardly possible that all action is suspended, even under conditions of extreme dryness or low temperature. The fact that life finally ceases even under these conditions of apparently complete suspension in itself proves that there are still changes taking place in the protoplasm, since the transition from the state in which it is capable of reproducing itself to the state in which this power is lost presupposes some chemical activity. The main consideration in preserving a culture is to attain a condition of dormancy as complete as possible, in order that the vital activities may be reduced to the lowest possible ebb without completely extinguishing them.

Four of the elementary necessities for maintaining life are moisture, heat, oxygen, and food. Obviously the latter becomes unavailable when moisture is withdrawn. The length of time a dry culture retains its vitality may be influenced by the degree of its dryness (in other words, the amount of moisture it contains), the temperature at which it is held, and possibly by the nature of the gas in which it is held.

INFLUENCE OF MOISTURE.

It is to be expected that the time a powder would retain its activity would be influenced by the amount of moisture it contained, since the life of the cell is dependent on a supply of water and its metabolic activity ceases when water is withdrawn. This was found to be true in an experiment conducted in the following manner:

A portion of a powder of fair activity was exposed overnight in a desiccator over sulfuric acid; another portion was exposed in a similar way for 3 hours over water. The original powder was found to contain 1.39 per cent of moisture, that exposed to sulfuric acid 0.90 per cent, and that held over water 5.77 per cent. The 3 lots were sealed in small phials and held in an incubator at a temperature of 28° C. At the end of 157 days 1 gm. of each lot was weighed with proper corrections for moisture content into flasks each containing 200 c.c. of milk, these were warmed to 30° C. and incubated at 30° C. overnight. The relative activity of the powders was measured by the titration of the milk at stated intervals. The results, given in Table 12, are averages of 2 flasks made on different days.

It will be noted that while there was no appreciable difference in the results with the powders containing 0.90 and 1.39 per cent of moisture, the powder with 5.77 per cent had fallen off distinctly in its activity.

To obtain the best results cultures should be dried as completely as possible and placed at once in a package that will exclude the possibility of their absorbing moisture from damp air.

TABLE 12.

INFLUENCE OF MOISTURE CONTENT ON LOSS OF VITALITY.

HOURS FROM INOCULATION	ACIDITY IN MILK		
	0.90 Per Cent Moisture	1.39 Per Cent Moisture	5.77 Per Cent Moisture
	Percentage	Percentage	Percentage
0	.171	.171	.171
17:30	.279	.243	.234
19:30	.315	.351	.261
21:30	.342	.405	.297
23:30	.459	.549	.342

INFLUENCE OF TEMPERATURE.

It is almost an axiom that bacteria succumb to unfavorable conditions most quickly at temperatures at which they grow most rapidly. A lactic culture survives for a long time in a refrigerator but lives only a few days in an incubator. Accordingly we should expect that dried cultures would deteriorate more rapidly in a warm room than in a refrigerator.

Paul[1] dried staphylococci on garnets and exposed them at room temperature, in an icebox, and in liquid air. At the end of a 32-day-period the bacteria at room temperature had decreased from 90,800 to 300. At the icebox temperature the decrease was less rapid but was marked, while at the temperature of liquid air there was no appreciable change.

A number of tests were made to determine the relative rate of deterioration at different temperatures by holding parts of the same powder at the various constant temperatures available in the laboratory and, after long periods, determining the activity of the powder by adding a definite amount to milk and measuring the rate of acid formation. One milligram of the powder was weighed out and added to 1,000 c.c. of milk which was incubated at 30° C. and the activity titrated at the end of 17, 19, and 21 hours. The results of one of these, which is representative, are given in Table 13.

In 30 days a distinct loss in activity had taken place at the higher temperatures. In 60 days at 30° C. and 37° C. the bacteria were nearly or quite dead, and at 18° C. the deterioration was marked. At 0° and −6° C. there was some loss, but the powder was still very active.

Table 14 shows the results of another test in which the bacteria were determined in a powder after 128 days at various temperatures. The powder which was dried in a current of air, contained originally a little over 3,000,000 bacteria per gram. At 0° C. the loss was within the limits of error, but at 5° to 10° C. and at 17° C. there was a 30 per cent loss, while at 30° C. the decrease exceeded 60 per cent.

[1] *Biochem. Ztschr.*, 1909, 18, p. 1.

INFLUENCE OF ATMOSPHERE IN WHICH CULTURE IS HELD.

It is reasonable to suppose that dried cultures would be affected to some extent by the gas in which they are held. Many bacteria are more active in the presence of oxygen than in its absence. Marshall[1] has observed that carbon dioxid has a detrimental influence on some bacteria.

The relative rate of loss of vitality as influenced by different gases was determined by holding about 1 gm. of powder in tubes in which the air was displaced by the gases studied. One tube was sealed without displacing the air, and in one tube in each set the air was exhausted by a Geryk pump giving a vacuum of about 0.01 mm. In other tubes the air was displaced by oxygen, hydrogen, nitrogen, and carbon dioxid. The oxygen was obtained by dropping boiled distilled water slowly on solid sodium peroxid and washing the gas with water and drying with sulfuric acid.

TABLE 13.

INFLUENCE OF TEMPERATURE OF STORAGE ON LOSS OF VITALITY.

HOURS FROM INOCULATION	ACIDITY AFTER 30 DAYS AT						ACIDITY AFTER 60 DAYS AT				
	Fresh Culture	−6° C.	0° C.	18° C.	30° C.	37° C.	−6° C.	0° C.	18° C.	30° C.	37° C.
	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age	Per-cent-age
17	.362	.369	.329	.257	.156	.149	.288	.274	.193	.180	.198
19	.495	.689	.779	.752	.424	.158	.355	.369	.193	.175	.193
21	.657	.....	.....	.....	.....	.....	.423	.553	.229	.175	.180

TABLE 14.

INFLUENCE OF TEMPERATURE OF STORAGE ON LOSS OF VITALITY.

AGE OF POWDER	BACTERIA PER GRAM IN POWDER HELD AT			
	0° C.	5–10° C.	17° C.	30° C.
Initial	3,270,000	3,270,000	3,270,000	3,270,000
128 days	3,180,000	2,420,000	2,500,000	930,000

Hydrogen was obtained by running dilute hydrochloric acid on chemically pure zinc. The gas was purified by passing through a solution of lead acetate and over broken earthenware covered with a paste of silver sulfate and dried by passing over phosphorous pentoxid.

Nitrogen was obtained by drawing air (1) through concentrated sulfuric acid, (2) through alkalin pyrogallol, (3) over heated copper oxid, (4) over heated copper spiral, (5) through potassium hydrate, and (6) over calcium chlorid.

The carbon dioxid was made by dropping dilute sulfuric acid slowly on sodium carbonate, and purified by passing through water and sulfuric acid. The tubes in which the powder was held were made with a bulb in the middle to hold the powder and capillary tubes at each end, one of which was necessarily sealed on after the tube

[1] *Centralbl. f. Bakteriol.*, 1902, 9, pp. 313, 372, 429, and 483.

was filled. The gas was passed through for 1 to 2 hours and the capillary tubes were sealed while the gas was still flowing.

Several sets of tubes were held in this way under varying conditions. The results of two of these experiments are given in Table 15, which shows the number of bacteria in each gram of the powder before and after the exposure to the different gases, and Table 16, in which are tabulated the results obtained from another lot by inoculating the powders into milk.

TABLE 15.

INFLUENCE OF VARIOUS GASES ON LOSS OF VITALITY.

HELD IN ATMOSPHERE OF	BACTERIA PER GRAM	
	Initial	After 132 Days
Air	3,270,000	750
Oxygen	3,270,000	7,750
Hydrogen	3,270,000	350,500
Nitrogen	3,270,000	22,000
Carbon dioxid	3,270,000	276,500
Vacuum	3,270,000	785,000

TABLE 16.

VIABILITY OF DRIED CULTURES HELD IN VARIOUS GASES.

Acidity of Milk Inoculated with Cultures after 30 Days' Exposure at 30° C., One Part Powder to 40,000,000 Parts Milk.

TIME FROM INOCULATION	ACIDITY OF MILK INOCULATED WITH CULTURE HELD IN					
	Air	Oxygen	Nitrogen	Hydrogen	Carbon dioxid	Vacuum
	Percentage	Percentage	Percentage	Percentage	Percentage	Percentage
15 hours	.175	.162	.189	.207	.162	.180
19 hours	.166	.162	.175	.283	.162	.202
21 hours	.162	.189	.198	.450	.166	.301
23 hours	.....	.261	.360	.589	.175	.625
SAME POWDER AFTER 45 DAYS AT 30° C. ONE PART POWDER TO 1,000,000 PARTS MILK.						
18 hours	.171	.144	.144	.171	.144	.252
20 hours	.171	.144	.144	.171	.144	.549
24 hours	.171	.144	.189	.396	.162	.702

There are some discrepancies in these results as to the relative loss of vitality in different gases, but they all agree in showing the greatest losses in air and in oxygen and the greatest activity in the powder held in the vacuum. The activity of the powder held in the inert gases, hydrogen and nitrogen, was usually greater than that held in carbon dioxid, and always decidedly greater than that held in air or oxygen, but less than in the vacuum tube. It will be noticed that Table 15 shows more bacteria in the powder held in

TABLE 17.

COMPARATIVE VIABILITY OF BULGARICUS POWDERS IN AIR AND IN VACUUM.

AGE OF CULTURE	HELD IN AIR					HELD IN VACUUM				
	Hours					Hours				
	17	19	21	23	25	17	19	21	23	25
Days	Percentage of Acidity					Percentage of Acidity				
0.....	.225	.351	.441	.486	.....	.225	.351	.441	.486	.....
7.....	.189	.288	.342	.504	.....	.189	.270	.351	.540	.....
14.....	.162	.189	.234	.270	.....	.189	.207	.243	.324	.....
21.....	.144	.189	.279	.396	.....	.171	.243	.351	.513	.....
28.....	.189	.216	.288	.342	.....	.207	.297	.414	.603	.....
42.....	.171	.....	.234	.333	.....	.216	.....	.387	.621	.....
101.....	.171	.171	.....	.171	.216	.216	.288	.....	.342	.486

carbon dioxid than in that held in nitrogen. In all other cases, however, the powder held in carbon dioxid was weaker than those exposed to nitrogen or hydrogen. It is difficult to explain the greater decrease in activity in the tubes filled with air than that in tubes filled with the individual gases occurring in air. It is possible that the air, which was inclosed without drying, may have held more moisture than the other gases, which were carefully dried, and this additional moisture may account for the difference in the effect on the powders.

B. bulgaricus cultures held in a vacuum show an activity distinctly greater than parts of the same powder held in air. This is illustrated by the results shown in Table 17, which were obtained by sealing portions of a *B. bulgaricus* powder in small phials and holding at room temperature with similar portions from the same powder which were held in sealed evacuated tubes. A definite amount of the powder was added to one liter of milk which was incubated at 37° C. and the acidity determined at 17, 19, 21, and 23-hour periods.

In 42 days the cultures in air were perceptibly weakened while those in vacuum were apparently

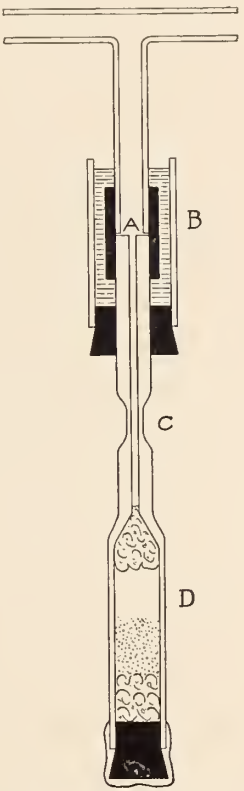

FIG. 3.—Apparatus for evacuating small tubes of powder.

unchanged. This observation is utilized in preparing cultures for distribution. For this purpose tubes are made as shown in Fig. 3. The powder is placed in Tube *D* between cotton plugs, a stopper inserted and covered with a cement made by boiling together resin, gum rubber, and a little vaseline. Connection between these tubes and the pump is made by means of a glass tube with a number of side arms. The joint at *A* is made by a rubber tube, inclosed in a glass tube, *B*, held in place by a rubber stopper. This tube is filled with mercury so that the rubber tube is covered. After the evacuation is complete the tube is sealed off at the constriction, *C*.

SUMMARY.

Commercial dry cultures of the lactic acid bacteria usually contain a small number of lactic bacteria and a high contamination, a condition due to crude methods of drying and to the loss of vitality of the bacteria on standing.

When cultures are dried by exposure to a current of warm air there is a constant decrease in the number of bacteria, which is greatly accelerated when the moisture is reduced to about 10 per cent. When a water content below 5 per cent is reached the decrease becomes very slow.

The loss in drying is greatly reduced when the water is removed very rapidly, as by the spraying method.

Water may be removed from cultures by exposing them in a frozen condition over sulfuric acid in a vacuum approximating 0.01 mm.

Lactic cultures dried by this method are sufficiently active to curdle milk in 17 hours at 30° C. when added in the ratio of 1 part of powder to 1,000,000 parts of milk.

The total number of bacteria in a milk culture may be increased by adding dibasic potassium phosphate, but the powder made from the culture is less active than that made from unneutralized milk. The activity of the powder is not increased by neutralizing the culture with calcium carbonate before drying.

More powder may be produced at each operation of the dryer by using a culture grown in concentrated milk, and this powder is as active as that made from normal milk.

Very active dried cultures of *B. bulgaricus* may be made by the freezing-vacuum method.

Laboratory cultures may be preserved by drying small quantities in test tubes.

The nitrogen-fixing bacteria may be dried with a small loss by the freezing-vacuum method.

Yeasts evidently do not survive the process, and the powders obtained were very feeble.

The loss of activity in powders is much more rapid when the moisture content is comparatively high.

The loss in activity is very slow at 0° C. or lower, and becomes more rapid as the temperature is increased. Dried cultures of the lactic-acid bacteria held at 30° or 37° C. become inactive in a short time.

Cultures held in a vacuum retain their activity much better than cultures in an atmosphere of nitrogen or hydrogen; the most rapid loss of activity takes place in an atmosphere of oxygen or air.

A SEPTIC SORE THROAT EPIDEMIC IN CORTLAND AND HOMER, N.Y.*

CHARLES E. NORTH, BENJAMIN WHITE, AND OSWALD T. AVERY.

I. HISTORICAL.

Since 1875 outbreaks of sore throat have been reported in England. A number of large epidemics have been officially investigated there, in all of which the symptoms of the disease have been characteristic and the relationship to the milk supply either suspected or confirmed.

Swithinbank and Newman[1] state that "in all probability these outbreaks are comparatively common, but, as the condition is not notifiable, it is seldom that a record is obtained of the cases occurring. We think it is safe to assume that a year never goes by in which there are not outbreaks of sore throat or tonsillitis due to milk or cream. The usual symptoms are congestion of the tonsils and mucous membrane of the throat, with sometimes ulceration, enlargement of the cervical glands, and some pyrexia and general malaise." The authors suggest some relation between scarlet fever and this disease, but believe they are not identical. They tabulate 9 typical outbreaks. Among these they mention an outbreak at Dover in 1884, affecting 205 persons, all of whom obtained milk from one dairy farm. The chief symptoms were local inflammations of the throat and enlargement of the lymphatic glands of the neck. In 1890 they mention an outbreak at Craigmore affecting 80 persons. As secondary to the sore throat, a number of cases of erysipelas developed. The incubation of the disease was 3 or 4 days. They record an outbreak in April and May, 1900, at North Hackney, which included 151 cases, 138 of which were supplied with milk from one dairy. The symptoms included tonsillitis, swelling of the cervical lymphatic glands, rise of temperature—in some cases to 105° F.—and great prostration. Secondary to the sore throat, in 1 case septicemia developed, followed by pneumonia and death; in 2 cases, acute nephritis. The authors state that there was a marked tendency for multiple cases to occur in families. Another outbreak mentioned by these authors occurred at Bedford, in June, 1902, including 42 cases. In every case milk was obtained from the same dairy. The health officer reporting this outbreak states: "I think that every case of septic sore throat should be notifiable. The difficulties of tracing an outbreak like this to its origin in the dairy can only be successfully carried out by a sanitary expert with the aid of a skilled bacteriologist." He also states: "Milk which was boiled did not produce the disease, but cream, which could not be boiled, produced the disease." In many of these sore-throat outbreaks the investigators found dairy cows suffering from mastitis, and they reported this inflammation of the udder as the probable cause. The bacteria most commonly found in the throats and udders were streptococci.

* Received for publication November 15, 1913.

[1] *Bacteriology of Milk*, New York, 1903, p. 352.

In the United States this disease has attracted much attention in the last 3 years owing to 3 extensive outbreaks.

The first of these, reported by Winslow,[1] occurred in Boston, Massachusetts, in the winter of 1910–11. The total number of cases collected was 1,043. The epidemic lasted over a period of about 2 weeks in Boston, Brookline, and Cambridge. About 70 per cent of the cases were supplied by one dairy. In the statistics reporting these outbreaks Winslow concludes that the milk from this dairy was the means of transmission of the disease, but that it was infected by human beings and not by dairy cows. Altho there were no acute cases among dairy employees the author states: "A carrier case presumably infected the milk."

In December, 1911, and January, 1912, an extensive outbreak occurred in Chicago. In the report by Capps and Miller[2] the authors estimate that over 10,000 persons were victims of the outbreak, the majority of whom were taking milk from the same dairy. They state that an epidemic of mastitis occurred among the cows supplying milk to this dairy during the winter months. Dr. D. J. Davis,[3] of Chicago, reports, concerning the examinations of the milk, that they found a streptococcus identical in morphology, in culture, and in pathogenicity with a culture obtained from a human case of tonsillitis and arthritis. E. C. Rosenow[4] reports further studies of this streptococcus and other streptococci from milk. The author is not positive in his conclusions, however, for he states: "The fact that milk so modifies streptococci is an additional indication of the important part it may play in epidemic sore throat. It is not possible to determine whether the streptococci in such epidemics are of exclusively bovine or human origin; they may be both. Milk drawn in a sterile way from normal cows may contain virulent streptococci and pneumococci; hence, "certified milk," while surely less contaminated than ordinary milk, may contain pathogenic bacteria, and the advisability of pasteurization even in this case should be considered, especially during the season when sore throat is common."

In Baltimore an epidemic occurred in 1912 during the months of February and March. This was reported by several authors, the most extensive report being made by Frost and Stokes and Hachtel.[5] A total of 602 cases was reported, the majority of which occurred from February 1 to March 20, 1912. The symptoms included sudden onset with chill, irregular fever, inflammation of tonsils, enlargement of cervical glands, and unusual prostration. Of these cases 65 per cent were supplied with milk from one dairy. This dairy supplied only 3.3 per cent of the milk sold in Baltimore. There were 28 deaths following the sore-throat attacks due to secondary infection, which included 13 cases of peritonitis, 7 cases of septicemia, 1 case of appendicitis, 2 cases of erysipelas, and 4 cases of pneumonia. The milk was pasteurized by the flash method. The author states: "Either the pathogenic germs were introduced into the milk by some infected person who had had to handle the milk, or were derived from the udders of some of the cows which produced it." The latter is believed to be the more probable hypothesis, altho no evidence could be obtained of the epidemic among the cows, and the veterinary examination of the cows through March and April failed to discover any cows with mastitis.

[1] *Jour. Infect. Dis.*, 1912, 10, p. 73.

[2] *Jour. Am. Med. Assn.*, 1912, 58, p. 1111; *ibid.*, p. 1848.

[3] *Ibid.*, p. 1852.

[4] *Jour. Infect. Dis.*, 1912, 11, p. 338.

[5] *Pub. Health Report 47, U.S. Public Health Service*, 1912, 27, p. 1889.

Stokes and Hachtel recovered pneumococci from the milk, showing that the flash system of pasteurization was inefficient. In the raw milk shipped to Dairy 1, they isolated a streptococcus of the epidemicus type. Several gargety cows were found among the dairy herd. The authors state: "We feel reasonably sure that the infection was caused by streptococci of the epidemicus type from cases of mastitis among the herds supplying this dairy."

In February, 1912, there was a sudden increase in the acute infections noted in Boston, almost a year later than the great Baltimore outbreak previously described. In this outbreak, which was investigated and reported by Coues,[1] 227 cases were reported. Forty-eight and five-tenths per cent were found to be taking milk from one dairy. Examination of the employees in the plant of this dairy showed that 16 men had remarkably reddened throats. A visit to the dairy farm from which the milk was obtained showed 10 farm hands with reddened throats. The milk was not pasteurized. The author concludes: "The occurrence of so many cases of milk pharyngitis . . . . in dairymen . . . . and the presence of streptococci in so many of their throats is of great interest. . . . . It seems fair to state from clinical examination of 77 cases that there is a condition which might be termed dairymen's or milk handlers' pharyngitis, incident to the long hours of constant work on wet floors often covered with ice and milk. . . . . It would seem as if direct, or nearly direct, hand contact with the milk is impossible to avoid in some stage of the preparation."

From a survey of the outbreaks outlined above it is obvious that, with the exception of those reported from Boston, there is a tendency on the part of all observers to conclude that the source of infection in septic sore throat is to be found in the inflamed udder of the dairy cow and is due to the streptococcus which is discharged from the inflamed udder into the milk, through which it is transferred to the throats of milk consumers, but that such conclusions are based on circumstantial evidence and that positive and direct evidence is lacking.

II. CORTLAND-HOMER OUTBREAK.

During the months of February and March, and the first three weeks of April, 1913, while there were a few scattered cases of sore throat among the inhabitants of the city of Cortland and the village of Homer, New York, no unusual conditions were noted. In the last week of April, however, the physicians in both these communities were alarmed by the sudden increase in the number of their patients complaining of sore throat. Within 3 days the cases multiplied rapidly into a widespread epidemic of sore throat. The disease manifested itself by redness and swelling—a raw beefsteak appearance—of the fauces, a rise in temperature,

[1] *Am. Jour. Pub. Health*, 1912, 2, p. 419.

enlargement of the lymphatic glands of the neck, and, in some cases, great prostration.

The city of Cortland has a population of 12,000 and the village of Homer, a population of 2,700. They are 2 miles distant from each other. As soon as the outbreak was recognized, a special meeting of the Cortland County Medical Society was called and a committee appointed to study the epidemic. The health officers of Cortland and Homer also began an official investigation of the outbreaks in these communities. By the first week in May there were several hundred persons in these two communities who were suffering from the disease, altho only a few cases had been officially reported to those studying the outbreak. The milk supply of one dairy soon fell under suspicion, and Doctor Ball, the health officer, took prompt action by making an inspection of the dairy, isolating from the main herd two cows showing physical signs of udder inflammation, and ordering that their milk be no longer used. This milk dealer was ordered to suspend the delivery of all milk from his dairy until the completion of the investigation, and a supply of pasteurized milk was secured for him from a large pasteurizing station located in the neighborhood.

On May 7, an offer made by Doctor North to act as a special investigator was accepted by the committee of the Cortland County Medical Society and by the health officers of the two communities. Every facility was furnished by the local physicians and health officers for a complete study.

At a special meeting of the Boards of Health of Cortland and of Homer, regulations were passed declaring septic sore throat to be a contagious disease, reportable and quarantinable, and statements were published in the local papers urging citizens to avoid contact with those suffering from the contagion.

1. Organization of Investigation.

The investigation was greatly facilitated by the ready co-operation which was secured from the following bodies:

1. Public Health Committee of the Cortland County Medical Society.
2. Boards of Health of Cortland and Homer.
3. New York Milk Committee.
4. Hoagland Laboratory, Brooklyn.
5. DeLaval Separator Company.
6. New York Dairy Demonstration Company.

Through these agencies it was possible to organize thoroughly all branches of the investigation at once, and to carry on several parallel lines of work at the same time. The work fell into the following divisions:

a) Reporting of cases.—Printed blanks were prepared having spaces for residence, name, age, sex, type of disease, date of onset, date of first visit, number of adults in the family, number of children, milk dealer, and remarks. These blanks were distributed to all of the physicians in Cortland and Homer, and they were asked to record in this regular manner all of the cases occurring in their practice, and to report same promptly to the director of the investigation. Twenty-seven physicians in the city of Cortland and 5 in the village of Homer made reports in the manner indicated.

b) Detailed investigation of cases.—Two statisticians were furnished by the New York Milk Committee who made a house to house canvass both in Cortland and Homer, calling on physicians and patients to check up and confirm details regarding each report in order that the same might be complete.

c) Cultures taken.—Doctors Benjamin White and Oswald T. Avery at the Hoagland Laboratory in Brooklyn made bacteriological examinations of cultures, taken from sore throats and from the sore udders of dairy cows.

d) Dairy inspections.—Early in the investigation, it became evident that a much larger number of cases were the patrons of one milk dealer than his proportion of the milk trade would warrant. Inspection was made of his own dairy and of another dairy from which he occasionally purchased a small quantity of milk. Physical examinations were made of dairy cows by the local veterinarian.

e) Sediment tests of cows' milk.—A new form of centrifuge called the Clarifier was furnished by the DeLaval Separator Company, with an expert operator, and proved of the highest value in the examination of cows' milk for inflammatory products. The apparatus was installed in the dairy barn of the dairyman under suspicion, and the milk of each dairy cow was examined by this means. From those cows in which the presence of inflammatory products was obvious, cultures were sent to the laboratory for bacteriological examination.

f) Pasteurization of milk.—The New York Dairy Demonstration Company in Homer consented to carry out the washing and sterilizing of all milk utensils of the suspected dairyman and furnish him with pasteurized milk for his retail trade during the progress of the investigation. All of his own milk supply was taken out of the market and disposed of for manufacturing purposes.

g) Inquiries in other towns.—To determine whether the outbreak was strictly local in character or not, inquiries were carried out in nearby towns and villages.

h) Tabulation of results.—As fast as the detailed reports of cases were completed, the results were tabulated at the central office by the director of the investigation, the entire local work occupying a period of about one week.

2. CASES REPORTED.

The cases reported by the physicians of Cortland and by the physicians of Homer were tabulated. Because of the obviously large percentage of cases occurring among the customers of one

dairy, which will be called Dairy X, the number of cases occurring among the patrons of this dairy were separated from those occurring among the patrons of all other dairies. Since the outbreak occurred simultaneously in the communities of Cortland and Homer, and in both of these a considerable number of cases was found among

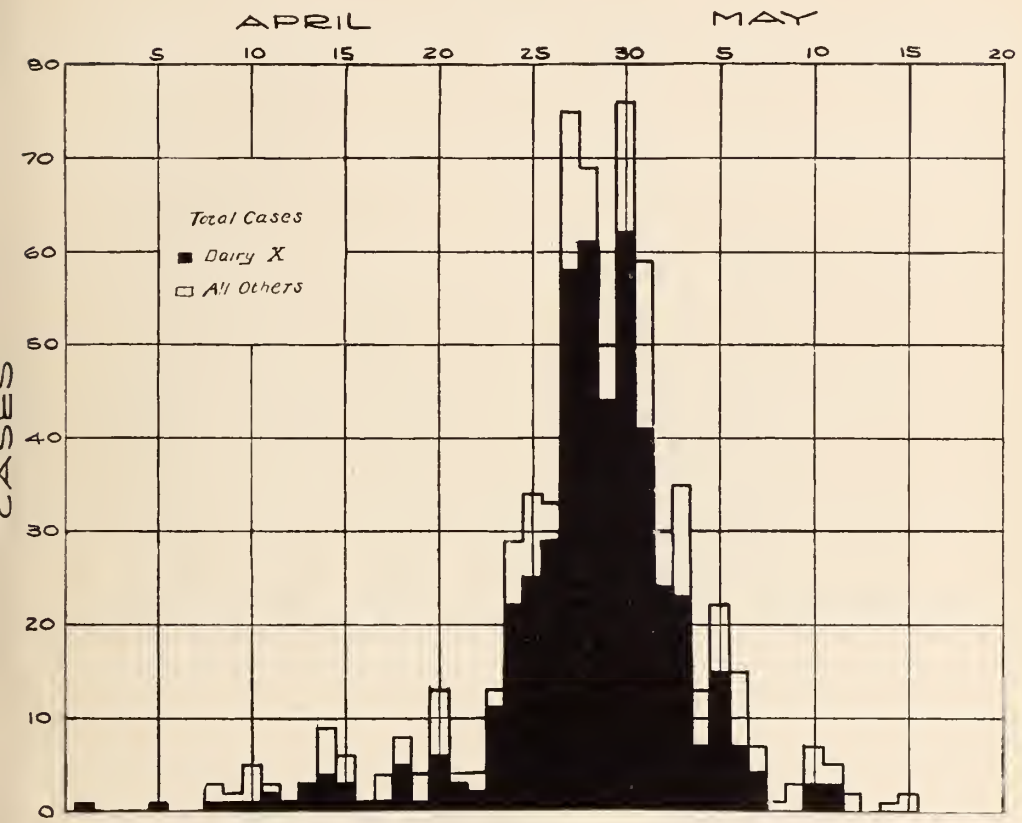

Chart 1.

the patrons of Dairy X, the figures for both Cortland and Homer are combined in Table 1, in order to secure a comprehensive idea of the entire outbreak.

The rise and fall of the cases in Cortland and Homer and of the cases found among the patrons of Dairy X is shown graphically in Chart 1. From this chart it appears that the epidemic

TABLE 1.

Date	Total Cases	Dairy X	Other Dairies	Date	Total Cases	Dairy X	Other Dairies
April				April			
1	1	1	0	21	4	3	1
2				22	4	2	2
3				23	13	11	2
4				24	29	22	7
5	1	1		25	34	25	9
6				26	33	29	4
7				27	75	58	17
8	3	1	2	28	69	61	8
9	2	1	1	29	50	44	6
10	5	1	4	30	76	62	14
11	3	2	1	May			
12	2	1	1	1	59	41	18
13	3	3	0	2	30	24	6
14	9	4	5	3	35	23	12
15	6	3	3	4	13	7	6
16	1	1	0	5	22	15	7
17	4	1	3	6	15	7	8
18	8	5	3	7	7	4	3
19	4	1	3	8	1	0	1
20	13	6	7	9	3	0	3

extended over a period of about 20 days, beginning April 20 and ending May 10, and that its crest was reached about April 30.

The figures given show the number of persons living in houses to which the milk of Dairy X was delivered. All of these persons were not milk drinkers. The proportion of cases found in householders receiving milk from Dairy X was so great that it was thought to be unnecessary to make a detailed inquiry as to those persons who did or did not drink milk. For the same reason a tabulation of the number of persons who lived in the houses to which milk was delivered who did not become victims of the disease was not undertaken. The facts shown in Table 1, and their relation to the milk supply of Dairy X, are summarized in Table 2.

TABLE 2.

	Cortland	Homer	Totals
Population	12,000	2,700	14,700
Total cases	483	186	669
Dairy X	360	120	480
Other dairies	123	66	189
Milk dealers	14	5	19
Milk supply	4,200 qts.	700 qts.	4,900 qts.
Dairy X supply	300 "	50 "	350 "

From these summaries it appears that about 72 per cent of the cases were found among the patrons of Dairy X, while, on the other hand, Dairy X was delivering less than 7 per cent of the total milk

supply. This means, in short, that there were 10 times as many cases of septic sore throat among the patrons of Dairy X as that dairy would be entitled to, had the cases been evenly distributed among all milk dealers.

TABLE 3.

Age Distribution of Cases.

Ages	Cortland	Homer	Ages	Cortland	Homer
0–5	41	8	30–40	93	31
5–10	42	10	40–50	61	24
10–20	76	29	50–60	36	19
20–30	91	24	60+	37	42

The age distribution of the cases is shown in Table 3. The occurrence of a larger number of cases between the ages of 20 and 40 than at other times of life corresponds to conditions found in other similar outbreaks.

Sex distribution was also recorded, showing that in Cortland there were 215 males and 268 females suffering from the disease. In Homer 71 males and 115 females were afflicted.

3. DEATHS.

There were 14 deaths in all reported by the local physicians as resulting from this epidemic. In each case death was due to complications. The date, age, and complication causing death have been separately tabulated in Table 4.

TABLE 4.

Deaths.

Date	Age	Cause of Death
	Cortland	
May 3	50	Edema of larynx
" 4	51	Heart failure
" 5	69	Lobar pneumonia
" 11	39	Peritonitis
June 14	71	Erysipelas
	Homer	
May 3	66	Peritonitis
" 3	60	"
" 4	62	"
" 5	70	Erysipelas
" 6	82	"
" 7	80	Pneumonia
" 8	72	Heart disease
" 11	78	Erysipelas
" 25	.....	Peritonitis

Of the 14 deaths, 5 were due to peritonitis, 4 to erysipelas, 2 to pneumonia, 2 to heart failure, and 1 to edema of the larynx.

In addition to the complications shown in the list of deaths, the physicians have reported that a considerable number of persons contracted rheumatism which manifested itself chiefly in the joints. In some cases the rheumatic symptoms were severe. There were also several cases of erysipelas, in addition to those resulting in death. Pleurisy and pericarditis have also been mentioned by some physicians as complications.

Inquiries were made in the communities located nearest to Cortland and Homer. These were the small villages of Preble, Scott, and McGraw, having populations of from 400 to 1,000, and located from 3 to 5 miles distant. The health officers of each of these villages stated that there were no cases of septic sore throat occurring in those places during the period of this outbreak. It is of further interest to note that no milk was supplied to these villages by Dairy X.

4. DAIRY INSPECTION.

Before the investigation was fully organized the health officer of Cortland, with a local veterinarian, had made physical examinations of the dairy cows in the herd of Dairy X and another dairy herd from which the proprietor of Dairy X occasionally purchased milk. As a result of this examination, Doctor Ball ordered two of the cows in the herd of Dairy X, showing external physical signs of inflammation, to be separated from the rest of the herd. Inspections of these two herds were again made and physical examinations of the dairy cows were duplicated by the local veterinarian, with the same result as in former examinations.

In the case of the other dairy, duplicate examinations failed to reveal any animals with udder inflammation. As later facts showed that the two cows having inflamed udders in the herd of Dairy X were undoubtedly responsible for the epidemic of septic sore throat, to the prompt action taken by Doctor Ball, the health officer of Cortland, must be credited the rapid decline in the number of new cases of sore throat which immediately followed.

For the first time in the history of the investigation of individual cows for the existence of udder inflammation, a new form of apparatus, the DeLaval milk clarifier, was used. The herd of Dairy X numbered 28 cows. The milk at the night's milking of all animals was examined by means of this apparatus. The machine was cleansed and sterilized between each operation by taking the apparatus entirely apart and washing and brushing all parts with a strong soda solution, after which they were rinsed in clean water. They were then plunged into a 1 per cent solution of chlorid of

Fig. 1

lime, and afterward thoroughly rinsed in boiling water. This procedure was followed between the examination of each cow's milk. Two men were thus able to secure the sediment from the milk of about 9 cows at one milking, and it required three afternoons to secure sediment from every member of the herd.

In Fig. 1 are shown typical results of these examinations. The glass bottles are 2 ounces in capacity. The first 3 show the quantity of sediment obtained from the milk of 3 dairy cows which were apparently normal. The quantity of milk produced by these animals at the night's milking varied from 6 to 8 quarts each. The

last 2 bottles contain the sediment obtained by the clarifier from the milk of the 2 animals in which udder inflammation was suspected. In one case, the quantity of milk produced was 1 quart, and in the other, 1.5 quarts. The difference in the quantity of sediment secured from their milk as compared with sediment found in the milk of normal animals is too obvious to require comment.

In these examinations, the use of the clarifier made it possible to eliminate the existence of udder inflammation from other members of the dairy herd, and to confirm most emphatically the existence of udder inflammation in the 2 dairy cows under suspicion. The quantitative results alone furnished sufficient evidence for these purposes. There were, however, qualitative differences in the slime which were even more interesting, as shown by microscopic examinations.

5. MICROSCOPICAL EXAMINATIONS.

Smears were made on glass slides of all samples of slime as soon as they were obtained. Experience in examining milk sediments has shown that considerable change takes place in appearance, particularly of the tissue cells and bacteria, if sediments are kept for any length of time before examinations. The multiplication of bacteria alone is enormous. Degeneration of leukocytes and tissue cells is also very rapid. The best results are obtained, therefore, by making smears from samples which are as fresh as possible. In all of the sediments examined from this herd, there was shown, microscopically, no evidence of inflammatory conditions excepting in the cases of the 2 dairy cows already mentioned. The microscopical appearance of sediment from normal cows is shown in Fig. 2.

Here we observe a large number of cells, the majority of which are apparently of the same character. Many of these have multiple nuclei. It might be inferred that the cells were leukocytes or possibly pus cells. The more one studies sediments from milk the more must one hesitate, however, to call cells having multiple nuclei pus cells or even leukocytes. The cells shown in Fig. 2 are probably tissue cells, being epithelial cells shed in the normal process of lactation from the walls of the milk glands.

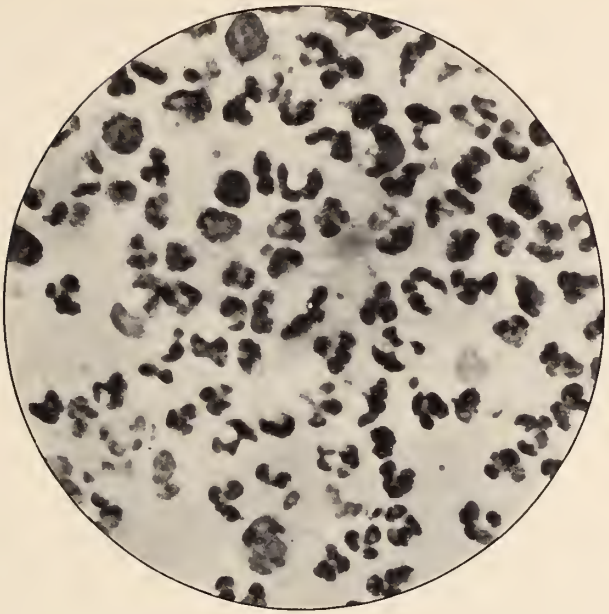

Fig. 2.

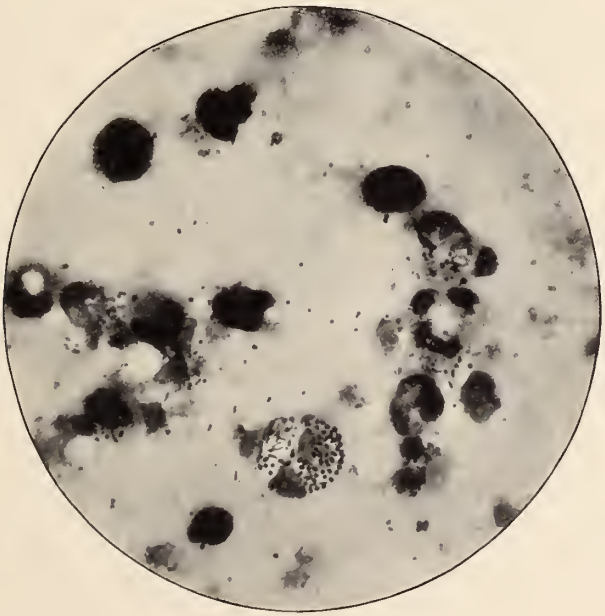

Fig. 3.

The microscopic appearance of the slime from the milk of the 2 cows having inflamed udders shows a marked difference in appearance. Fig. 3 shows a considerable variety of cells; of even greater interest are the large numbers of bacteria and the marked phagocytosis. No one would hesitate to say that cells must be pus cells which have multiple nuclei and are engaged in collecting such large numbers of bacteria within their bodies. The discharge from the udder into milk of streptococci, bacilli, and of phagocyting white blood corpuscles, and their appearance in milk sediment, must be accepted as evidence of the existence of udder inflammation. This condition was shown plainly in every smear which was made from the slime obtained from these inflamed udders.

6. BACTERIOLOGY.

A. ORGANISMS ISOLATED IN PREVIOUS EPIDEMICS.

The bacteriological examination of cultures from throat exudates in a number of patients and from the milk slime of gargety cows was undertaken with three objects in view: (1) to determine, if possible, whether any particular organism was common to all the cases; (2) to ascertain if any organism was common both to the throat exudates and to the slime; and (3) to study and identify any organisms found which were believed to be of significance.

As a result of studies in previous epidemics it is now generally held that the streptococcus is the chief etiological factor in septic sore throat. Further, the predominance of organisms of the streptococcus type in the inflamed udders of garget cows has drawn attention not only to the possible significance of this organism, but has suggested the probable original source of the human infection. In addition to this primary infection of the milk, another possibility presents itself in the chance of an accidental introduction into the milk of pathogenic organisms from individuals suffering from streptococcic infection and handling the milk.

In the Finchley epidemic in 1904 the streptococcus was first isolated from the throats of the affected patients, while *Str. pyogenes* was first detected in the suspected milk by Pierce[1] in the Guildford outbreak in 1903. Since that time, in the majority of outbreaks,

[1] *Brit. Med. Jour.*, 1903, 2, p. 1492.

the streptococcus has been isolated either from the throats of patients or from the milk supplied to the homes where infections developed.

The more careful studies of the latest outbreaks seem to strengthen the probability that the specific organism is a streptococcus resembling in many respects, but in others differing from, the pyogenes type. On account of its variations it has been classified separately as *Str. epidemicus*. The salient characteristics of *Str. epidemicus*, as described by Davis, Hamburger,[1] and Stokes and Hachtel, are as follows:

The organism is gram-positive and usually occurs in pairs existing separately or in short or longer chains. When freshly isolated, the pairs show a capsule, differing from the capsule of the pneumococcus in that it is not indented between the individuals in the pairs. It is frequently described as a halo and is rapidly lost on cultivation, but reappears after animal passage. On blood agar, according to Davis,[2] the colonies are larger and more moist than those of *Str. pyogenes*, but they do not have the mucoid appearance of *Str. mucosus*. The colonies are surrounded by a distinct zone of hemolysis, generally, but not always, narrower than the zone produced by *Str. pyogenes*, and exhibiting a faint greenish tint by transmitted light. *Str. epidemicus* is only partially dissolved by bile, and it acidifies milk with frequent but not constant coagulation. Dextrose, lactose, saccharose, and dextrin are fermented with acid production but no gas, while raffinose, mannite, and inulin are not attacked. Broth is uniformly clouded. Davis states that the organism is highly virulent for mice, rabbits, and guinea-pigs. Stokes and Hachtel confirm its pathogenicity for mice. The organisms isolated during the Chicago and Baltimore epidemics correspond to the above description.

B. NATURE AND COLLECTION OF SPECIMENS (PRESENT EPIDEMIC).

The exudate from the throats of 13 patients and the pus from a suppurating cervical gland were removed with a sterile swab and planted on Löffler's medium. The tubes were incubated and shipped immediately to the Hoagland Laboratory. The milk slime, obtained from the 2 suspected cows in Dairy X by means of the clarifier, was drawn into sterile bottles and immediately forwarded. All material was examined upon its receipt.

C. PROCEDURE OF EXAMINING MATERIAL.

The outlined procedure was followed with all material: The swabs were rubbed over the surface of the culture, then shaken in a tube of serum broth which was incubated over night at 37.5° C. This broth culture was then plated on rabbits' blood agar by the Conradi method, and the plates incubated. Colonies were fished and those yielding suspicious diplococci or streptococci were isolated on blood agar, nutrose agar, and in ascitic broth. The milk slime was planted in serum broth and then plated and subcultured as above.

[1] *Jour. Am. Med. Assn.*, 1912, 58, p. 1109; also, *Bull. 263, Johns Hopkins Hosp.*, 1913, 24, p. 1.

[2] *Jour. Am. Med. Assn.*, 1912, 58, p. 773.

TABLE 5.

CULTURE	ORIGIN	CHAIN FORMATION	COLONIES ON BLOOD AGAR			GROWTH IN BROTH	SOLUBILITY IN BILE	HEMOLYSIS	LITMUS MILK	CARBOHYDRATES†					PATHOGENICITY	
			Size	Color	Hemolytic Zone					Dextrose	Lactose	Saccharose	Salicin	Raffinose		
CI/2′	Throat	Short	Medium	Green	Meth.* brown	Uniform turbidity	—	Meth. brown	x	x	x	x	x	x	Rabbit, suppurative arthritis	Group A
CII/2′	"	Medium	"	"	" "	Uniform turbidity	—	" "	x	x	x	x	x	x		
CIV/1	"	Short	Large	"	" "	Uniform turbidity	—	" "	a	x	x	x	x	x	Rabbit died, 7 days	
J	"	.....	Small	"	" "	Uniform turbidity	—	" "	x	x	x	x	x	x		
CIII/1	"	Medium	"	"	" "	Uniform turbidity	—	" "	a	x	x	x	—	x		Group B
CV/1	"	Short	"	"	No Color	Uniform turbidity	—	" "	x	x	x	x	—	x		
CV/3	"	"	Medium	"	Meth. brown	Granular sediment	—	" "	x	x	x	x	—	x	" " 28 "	
CVI/1	"	Long	Large	"	" "	Ropy sediment	—	" "	x	x	x	x	—	x	" " 8 "	
CVIII/2	"	Medium	Small	Faint green	" "	Uniform turbidity	—	" "	x	x	x	x	—	x		
CIV/1	"	"	Medium	Faint green	No color	Uniform turbidity	—	No Color	x	x	x	x	x	—		Group C
CV/3	"	Long	Small	Faint green	" "	Granular sediment	—	" "	a	x	x	x	x	—		
CVI/2	"	Short	Medium	Green	" "	Uniform turbidity	—	" "	x	x	x	x	x	—		
CVII/1	"	Long	"	Faint green	Meth. brown	Granular sediment	—	Meth. brown	x	x	x	x	x	—	" " 2 "	
K	"	Medium	"	Green	Hemolysis	Ropy sediment	—	Hemolysis	x	x	x	x	x	—		
CVII/1/x	Cervical gland	Long	"	Faint green	Meth. brown	Granular sediment	—	Meth. brown	x	x	x	x	x	—		

* Methemoglobin brown.

† There was no action in mannite, inulin, and dulcite.

TABLE 5.—*Continued.*

CV/2	Throat	Long	Large	Green	Meth.	brown	Ropy sediment	—	Meth.	brown	*x*	*x*	*x*	*x*	—	—		Group D
CVIII/1	"	"	Medium	"	"	"	Ropy sediment	—	"	"	*a*	*x*	*x*	*x*	—	—	Rabbit died, 7 days	
CIX/2	"	Medium	Small	"	"	"	Uniform turbidity	—	"	"	*x*	*x*	*x*	*x*	—	—		
23	Milk slime	Long	Medium	"	"	"	Ropy sediment	—	"	"	*x*	*x*	*x*	*x*	—	—	Rabbit, suppurative arthritis	
24	Milk slime	"	"	"	"	"	Ropy sediment	—	"	"	*x*	*x*	*x*	*x*	—	—	Rabbit died, 8 days	
K-2	Throat	"		"	"	"	Ropy sediment	—	"	"	*x*	*x*	*x*	*x*	—	—		
M128/1	Milk			"	"	"		—	"	"	*x*	*x*	*x*	*x*	—	—	Not pathogenic for mice	Group E
S128/4	Milk slime			"	"	"		—	"	"	*x*	*x*	*x*	*x*	—	—	Not pathogenic for mice	
M152/4	Milk			"	"	"		—	"	"	*x*	*x*	*x*	*x*	—	—	Not pathogenic for mice	
S152/4	Milk slime			"	"	"		—	"	"	*x*	*x*	*x*	*x*	—	—	Not pathogenic for mice	
S187/3	Milk slime			"	"	"		—	"	"	*x*	*x*	*x*	*x*	—	—	Not pathogenic for mice	
S189/1	Milk slime			"	"	"		—	"	"	*x*	*x*	*x*	*x*	—	—	Not pathogenic for mice	
S189/4	Milk slime			"	"	"		—	"	"	*a*	*x*	*x*	*x*	—	—		

Litmus milk: *x* = acid coagulation. *a* = acid production but no coagulation.
Carbohydrate reactions: *x* = acid coagulation, — = no action.

1. *Morphology.*—Observations were made on young cultures in ascitic broth. Both the Hiss and the Rosenow capsule stains were used.

2. *Hemolysis.*—One-half cubic centimeter of an 18-hour ascitic broth culture was added to 1 c.c. of a 5 per cent suspension of washed sheep and human erythrocytes, respectively. Readings were taken at the end of 2 hours' incubation, and over night at room temperature. This method is considered by the authors as yielding more definite evidence of hemolytic action than that obtained by observing the colonies on blood agar.

3. *Solubility in bile.*—One cubic centimeter of a 24-hour broth culture was mixed with 0.2 c.c. of sterile ox-bile. Readings were taken after 30 minutes and 2 hours at 37.5° C.

4. *Carbohydrates and milk.*—The carbohydrates were Kahlbaum preparations and were employed in the form of Hiss serum waters. Decreamed litmus milk and the carbohydrate media were inoculated with approximately 0.1 c.c. of an 18-hour ascitic broth culture. Viability of the cultures used for inoculation was tested each time by planting on nutrose agar. Readings were taken during 7 days' incubation.

5. *Pathogenicity.*—One cubic centimeter of an 18-hour ascitic broth culture was injected into the ear veins of rabbits weighing approximately 500 grams.

D. RESULTS.

Table 5 shows the general morphological, cultural, and biochemical characters of the organisms isolated.

The colonies of all the strains of streptococci isolated on rabbits' blood agar showed a greenish tint by transmitted light. They varied in size from pin points to aggregations somewhat larger than the usual *Str. pyogenes* colonies. The mucoid appearance produced by *Str. mucosus* was never observed. The growth in broth varied with the presence or absence of enriching ingredients, and experience showed the advisability of disregarding this feature as a point of differentiation. From careful observation of the form of the cocci when grown in ascitic broth, it would be difficult to distinguish any reliable diagnostic differences. The occurrence of the cocci in pairs was frequent, and usually these pairs were linked together in short and sometimes moderately long chains. The use of the Hiss and the Rosenow stains failed to reveal a true capsule. When freshly isolated from the body, particularly on the direct smears made from the blood or pus of the infected rabbits, a distinct encircling zone could be seen about the paired or chained cocci, but its appearance would scarcely warrant its being designated as a true capsule. None of the organisms in the series was appreciably dissolved by ox bile, and only one—and that from a cervical gland—

produced any hemolysis when added to a suspension of washed sheep or human erythrocytes.

The various streptococci isolated appear to form general groups when classified according to their carbohydrate reactions. Altho any classification of streptococci according to carbohydrate fermentation is acknowledged to be incomplete and unsatisfactory, yet, lacking more valuable methods, the following grouping is tentatively offered. All the strains acidified dextrose, lactose, and saccharose, all acidified litmus milk, but none fermented inulin, mannite, or dulcite; hence their behavior toward raffinose and salicin is taken as a basis for the grouping.

Group A.—Salicin and raffinose fermented. All produced the methemoglobin darkening when added to erythrocyte suspension, but none gave a true hemolysis. All strains appeared as diplococci in short or medium chains. One of the strains, "CI/1," produced a suppurative arthritis in a rabbit, and from the lesion the organism was recovered and proved to be identical with the strain inoculated. Strain "CIV/1" killed a rabbit in 7 days.

Group B.—Raffinose fermented but not salicin. All except one produced methemoglobin, but no true hemolysis. The morphology was the same as in Group A. Two rabbits injected with two of the strains died in 8 and 28 days, respectively, of coccidiosis with no sign of streptococcic infection. In connection with this group there may be noted the observation of Stokes and Hachtel that their strains of *Str. epidemicus* developed the ability to attack raffinose on cultivation.

Group C.—Salicin fermented but not raffinose. There were no significant morphological features. Three strains produced no change in the erythrocyte suspensions, 2 gave the methemoglobin reaction, while 1 strain, "K," produced true hemolysis. It is interesting to note that this strain was obtained from a suppurating cervical gland, and was the only one in the whole series producing hemolysis. From the throat of this case, 3 strains were isolated, one, "K-2," falling in Group D, while the others, "K-3" and "K-4," differed from all the other strains examined in their ability to attack mannite. Strain "CVII/1" killed a rabbit in 2 days. The

organism was recovered from the heart's blood and corresponded in all respects to the original strain.

Group D.—Neither salicin nor raffinose fermented. All produce methemoglobin but no hemolysis. All the organisms isolated from the milk slime from the infected cows fall into this group. These strains agree with the description of *Str. epidemicus* as given by Davis, and Stokes and Hachtel, with the single exception that, altho the colonies on blood agar had a greenish tint, none of these strains produced true hemolysis when added to red blood corpuscle suspensions. The members of Group C, like those of D, fail to attack raffinose, but do attack salicin. Inasmuch as other authors make no mention of the behavior of *Str. epidemicus* toward salicin, it is impossible to draw sharp and complete comparisons.

In connection with the present series of cultures, there attaches a pertinent significance to some observations, made by the authors earlier in the year, on cultures of streptococci obtained from milk slime. At the time, a preliminary attempt was made to ascertain the nature of the predominating organism in milk and milk slime from several cows suffering from infected udders. The whole milk and the milk slime obtained by the clarifier was examined by the procedure already described. Cows No. 128 and 152 had frank cases of garget, Cow 187 had a slight infection of one quarter, while Cow 189 was supposed to be a normal animal, altho the quantity and nature of the slime obtained from the milk of this cow strongly suggested the presence of inflammation. The organisms isolated from these 4 cows are included in Group E. It may be pointed out that, in the case of the 2 severely infected animals, suspicious streptococci were found in both the milk and the slime, while, in the case of the other 2 animals, these streptococci were present only in the slime. The great similarity between the reactions of the members of Group E and those of Group D is strikingly suggestive.

SUMMARY.

1. Two epidemics of septic sore throat occurred simultaneously in May, 1913, in Cortland and Homer, New York, the two communities being 3 miles apart.

2. Over 70 per cent of the cases in each community occurred among the patrons of a dairyman who was the only dairyman selling milk in both places, and who supplied less than 7 per cent of the total milk supply. Adjacent towns had no cases and, further, they received no milk from the suspected dairy.

3. Physical examination of the cows and microscopical examination of milk sediments showed the existence of acute udder inflammation in 2 cows in the herd of the suspected dairyman.

4. Bacteriological examination showed that cultures from the throats of 4 patients contained streptococci apparently identical with strains of streptococci obtained from the milk slime from the 2 cows suffering from garget. Cultures from the throats of 8 other patients contained streptococci of this same type but differing, by slight variations only, in their carbohydrate fermentations.

5. The streptococci isolated from the milk slime from the 2 garget cows and the throats of 4 patients in the present epidemic correspond in all respects to strains of streptococci isolated previously from 3 cows of another herd known to be suffering from garget, and from the milk slime from 1 cow supposed to be normal, but which gave an abnormal amount of slime.

THE ACTINOMYCES-LIKE GRANULES IN TONSILS.*

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In the crypts of tonsils one commonly finds masses of gray or yellow material, foul-smelling, and varying considerably in appearance. These masses are referred to as tonsillar plugs (*Pfröpfe*) and are usually said to consist of bacteria, epithelial débris, etc. If one examines them carefully it is seen that they are not all alike. Some are made up of small grayish masses or clumps of desquamated and often hyalinized epithelial cells, in which a few cocci and bacilli without any definite arrangement may be found. Others are composed of soft, yellowish, pasty or waxy material and occur in occluded or partially occluded crypts. Cholesterin crystals are commonly found in them, but bacteria are rare and often not present. At times small cysts appear. There may also be formed real abscesses, containing thick pus, which in their early stages yield on culture various bacteria, but later are often sterile.

A third variety of tonsillar masses differs decidedly from the masses mentioned above and it is these that I wish to discuss in this paper. They are small, definite, grayish bodies or granules, rather brittle in consistency, and reveal, on examination, a raylike structure resembling in some respects the granules of actinomyces. They have not been the subject of much interest nor have investigations in regard to their real nature been numerous. In most textbooks they are not even mentioned. In special articles they are usually referred to as mycelial masses or growths and they have been repeatedly mistaken for actinomyces, a point which I shall discuss later. Here I wish merely to state that while their raylike structure bears a very superficial resemblance to the granules of actinomyces, in reality they are entirely different and indeed are not even remotely related biologically to the actinomyces group.

The first mention of these bodies in the literature appears to be a statement by Johne[1] in 1881 who called attention to certain granules resembling actinomyces found

* Received for publication August 15, 1913.

[1] Quoted by Bostroem, *Beiträge z. path. Anat. u. z. allg. Path.*, 1891, 9, p. 1.

in the tonsils of swine. A detailed description of them is not given but he states that they are similar to actinomyces granules and perhaps identical. Bostroem[1] states that he was able to confirm Johne's work, but he likewise does not describe them in detail, saying simply that morphologically they correspond to actinomyces.

Ruge[2] seems to be the first to call attention to these bodies in human tonsils. He says that it is probable that they belong to the actinomyces group, but he maintains that they are not identical with actinomyces hominis. They differ from the latter in having larger granules and coarser filaments and the granules are firmer and more durable. Club-bearing ends were not found. He points out that these bodies are not stained by Gram stain, while real actinomyces granules are so stained. Cultivation was evidently not attempted. He considers it possible that they may have something to do with hypertrophy of the tonsils.

Jonathan Wright[3] describes a case in which he found raylike granules in a sectioned tonsil. He refers to Ruge's paper as the only one that he could find in the literature on actinomycosis of the tonsils. In a footnote he says, however, that he is unable to conjecture whether Ruge's cases were actinomycosis or not. He notes that Ruge referred to the granules as "actinomyces-like," but he himself does not appear to consider the possibility that these bodies, which he also found in the tonsillar crypts, might not be true actinomyces. He describes their raylike structure and fortunately gives some drawings. To anyone familiar with these bodies it is clearly evident from his Figs. 1 and 4 that he is not dealing with true actinomyces but with the mycelial masses resembling actinomyces. He speaks of the proliferation of epithelium lining those crypts which contain the granules. No attempt to cultivate the organisms was made nor did he study them in smear preparations.

Gappisch[4] reviews the previous work done on this subject and gives a good description supplemented with photographs of these growths. The raylike structure and brushlike ends at the margin of the granules are well shown as are also the masses of bacilli and cocci. No reference is made to the spirilla forms. He states definitely that these granules are not actinomyces and indeed he appears to be the first to make this unequivocal statement concerning these bodies. Evidently no attempt was made to grow the various organisms. A point of interest in this work is the statement that the structures may invade the tissues. In two of his cases (Cases 1 and 10) there is loss of crypt epithelium and the masses lie in abscess cavities in the follicles where, he says, they remind one of actinomyces abscesses.

In 1906 Miodowski[5] pointed out that these granules have nothing to do with true actinomyces. In 147 cases he found them 12 times. At autopsy in 9 cases he found them once and in 19 operative cases he found them 4 times. He makes the statement that they are slightly gram-positive, differing, in this respect, from Ruge's results. He says that this condition is in no way related to the pharyngomycosis of *B. Fränkel*. He would not deny that they might have something to do with hypertrophy but thinks that in hyperplastic tonsils the conditions of the crypts are favorable for the development of these masses. He never found them in the pharyngeal tonsil tissue tho he examined as many of these as of faucial tonsils. He gives some excellent colored plates, an examination of which convinces one that he is surely dealing with these actinomyces-like masses and not with true actinomyces.

[1] *Loc. cit.*

[2] *Ztschr. f. klin. Med.*, 1896, 30, p. 529.

[3] *Am. Jour. Med. Sc.*, 1904, 128, p. 74.

[4] *Verhandl. d. deutsch. path. Gesellsch.*, 1905, 9, p. 130.

[5] *Arch. f. Laryngol. u. Rhinol.*, 1906, 19, p. 277.

From this review of the literature it appears that only a few of the essential features of the morphology of these bodies have been described and that no attempt has been made to cultivate and isolate them and to study their pathogenic properties. It is also evident that they have been confused with true actinomyces by several writers, a confusion which at present should not exist.

MORPHOLOGY OF GRANULES.

The granules are found only in the crypts and are usually small grayish or grayish-yellow bodies whose size is variable. They are commonly about as large as a pin-head but they may be much larger or much smaller. Occasionally they attain the size of a split pea and are with difficulty forced from the crypt openings. On the other hand, they are often so small as to be visible only with a lens or microscope. In shape they are irregular, being often lobulated. They are quite brittle, readily breaking into smaller fragments which later may be difficult completely to disintegrate. Calcification may be present in the mass, thus forming the tonsil stones. They invariably have a foul and disagreeable odor, especially when crushed, an odor which corresponds very well with that so often detected on the breath of persons having diseased tonsils. These bodies are found usually near the outlet and in the more expanded portions of the larger crypts; they may occur, however, deep down near the base of the crypts. As to their frequency, I think it is probably true that every tonsil contains these bodies at times. They are discharged from the crypts from time to time and on removal of the tonsils they are readily forced out of the crypts. In order to give some idea of their frequency I may state that in a careful examination of 122 pairs of tonsils, chiefly from children, they were visible to the naked eye or readily

FIG. 1.—Section of actinomyces-like granule lying in a tonsil crypt. About one-half of the entire granule is shown. Hematoxylin and eosin stain. ×120.

seen with a hand lens in the crypts of 30 tonsils. When found they were usually present in both tonsils tho there were many exceptions. These were all extirpated tonsils and no doubt in their enucleation in some instances these bodies were forced out from the crypts and lost. Furthermore, in microscopic preparations one sees frequently in the crypts very small raylike granules which would not be visible by an examination such as was made in the above-mentioned series. There is no doubt, therefore, that

FIG. 2.—The brush-like ends which occur at the margin of the granules. The fusiform bacilli are arranged perpendicular to a central stalk. ×1000.

these granules are considerably more frequent in tonsils than the foregoing data indicate.

Microscopically they present a very characteristic raylike structure. Fig. 1 is a photograph made from a section through such a granule lying in a tonsil crypt. The raylike structure is especially conspicuous. Near the center the mycelial filaments run in various directions tho generally outward. Nearer the periphery they assume a more parallel arrangement, the transition

zone being distinctly noticeable in the figure. In the centers of the granules it is not uncommon to see lime deposits, vegetable particles, etc., and often here and there large crystals which are probably fatty acid in nature. The structure of these bodies can be readily brought out by placing them on a glass slide in a drop of methylene blue or dilute carbolfuchsin and then pressing down a cover glass gently in order to spread the mass. At the periphery with the low power, club-like structures appear, which with higher

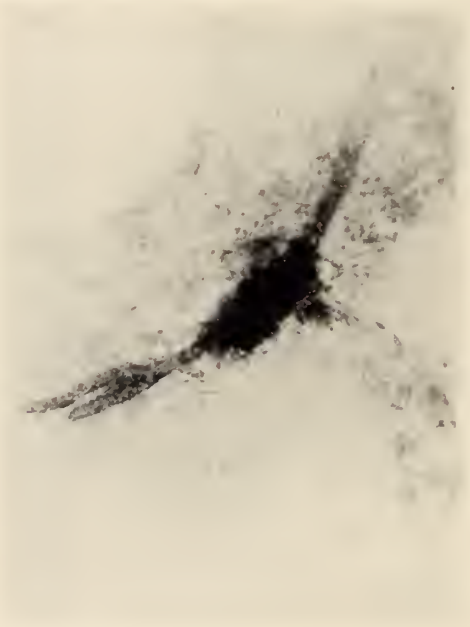

FIG. 3.—A branching stalk from which most of the fusiform bacilli have been separated. ×1000.

magnification are shown to have a somewhat complicated morphology. Such a structure is shown in Fig. 2. It consists essentially of a central shaft composed of mycelial filaments about which are arranged myriads of elongated bodies or bacilli vertical to the shaft. It may be compared to an ordinary test tube brush, the bristles of the brush corresponding to the bacilli and the wire shaft corresponding to the central filaments. The elongated bodies attached to the shaft are very easily detached by slight manipulation, as for example by making smear preparations, and then one

sees the stalks with few or almost no bodies attached. In Fig. 3 is shown a branched stalk from which the bodies have been separated except at a point farther down where some are still attached. The central stalk, it should be pointed out, is composed of one or commonly a bundle of filaments. The apparent branching is not a true branching of the filaments but simply a separation or division of the bundle. I have never seen true branching of the filaments or of the bacilli.

When the granules are carefully washed through several changes of sterile salt solution and smears are made therefrom by crushing, four kinds of organisms are always seen: namely, cocci, spirilla bacilli, and filaments.

The cocci belong to the streptococcus group. In smear preparations they occur at times singly, usually in pairs, rarely in short chains. They are strongly gram-positive. As a rule they grow rather feebly in aerobic cultures and somewhat better under anaerobic or partial anaerobic conditions. Some strains produce green color on blood agar, but as a rule they hemolyze slightly and often form a rather hazy zone about the colony. At times the zone may be clear and quite like that produced by the ordinary hemolytic streptococcus. These cocci when injected intravenously into rabbits may produce arthritis, but they possess, in general, low virulence and cause lesions only when given in large doses.

The spirilla are rather long and coarse (Figs. 4 and 5). They are gram-negative and stain readily tho faintly with ordinary dyes. Carbolfuchsin is a very satisfactory stain for them. They are always found in the smears, at times in enormous numbers. As yet no attempt has been made to study their cultural features.

In smear preparations of the granules the bacilli are seen mostly detached from the stalklike filaments, tho here and there if the smear has been carefully made there may be considerable numbers of them still adherent (Figs. 3 and 4). Usually they have pointed ends and stain unevenly; some show a definite barred appearance. Most of them are slightly curved. They stain readily with ordinary dyes and are weakly gram-positive. Too long washing in alcohol will decolorize them completely. Undoubtedly some workers would call them gram-negative. It may be said that they have

the morphology and staining properties of *B. fusiformis*. The filaments comprising the mycelial mass readily break up into longer or shorter threads. These are never branched and they do not contain septa. Often distinct granules are seen within the filament. They stain readily especially with carbolfuchsin and react to Gram stain in a manner similar to the bacilli above described. The relation of the filaments to the bacilli will be discussed later.

The cultivation of these bacilli is a matter of some interest, since, so far as the literature indicates, they have never been grown artificially. The following method was used successfully. The granule is thoroughly washed through several changes

FIG. 4.—Smear preparation of a granule showing fusiform bacilli, spirilla, and cocci. The fusiform bacilli predominate. A stalk is also shown to which a few fusiform bacilli are still attached. ×1200.

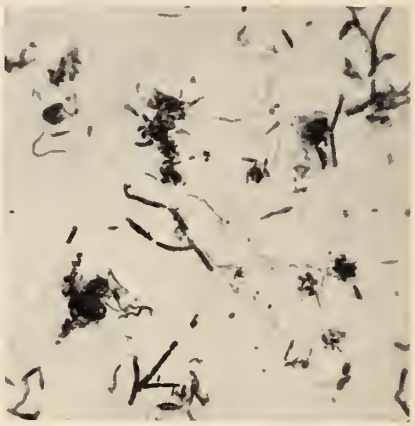

FIG. 5.—Smear preparation of granule showing large numbers of spirilla forms. Also some fusiform bacilli and cocci. ×1200.

of sterile salt solution in order to get rid, as far as possible, of surface contamination with mouth organisms. They are then crushed in a small amount of sterile fluid from which inoculation of suitable media is made. For this purpose 2 per cent glucose agar slants plus one-third volume of blood or serum are used. Several tubes are inoculated with varying dilutions and the oxygen is then removed by an alkalin pyrogallic mixture absorbed in a cotton plug at the top of the sealed tube. Cocci are always obtained and the colonies of bacilli must be differentiated—usually by their appearance and their slow growth. The strains vary in their ability to grow on artificial media. They are all strict anaerobes. At times no growth whatever appears; again good growth may appear on the first tubes and transplants fail absolutely. When the organisms have once passed through a few subcultures they then grow readily.

Of late I have used with success the method described by Krumwiede and Pratt,[1] which consists of a serum-sugar agar-plate culture the cover of which is imposed directly on the media in the plate in an inverted position. Thus there is a thin layer of media between the two glass surfaces. After 48 or 72 hours the plates are torn apart and the colonies then may be transferred to tube cultures.

For carrying along the cultures, serum or blood glucose agar slants or stabs are suitable. Serum gelatin agar is also suitable, tho it possesses no advantage over the serum glucose agar.

The colonies are barely visible on plates in 24 hours; after 48 hours they appear as small grayish colonies with somewhat irregular margins. They never become large and cease growing in size after about four or five days. On serum media they are gray; on blood media the color is dark brown or even reddish. On slants the growth when diffuse has a rough stippled appearance which does not penetrate below the surface. It is readily removed from the surface and forms without difficulty a fairly homogeneous suspension in fluid. On blood agar slant tubes the colonies will often grow in abundance between the glass and the media. Here they become quite large and may occur entirely around the tube and about the bottom, presenting a rather characteristic appearance against the dark-red background of media. In sterile tissue placed in the bottom of test tubes and covered with media pure cultures of the bacilli readily grow; they penetrate the tissues, causing a rapid disintegration with a fairly characteristic odor. No gas is produced in glucose media. They do not grow in plain broth, litmus milk, or on potato.

It may be stated that these organisms never form colonies or granules on artificial media that in any way resemble the granules in the tonsils. If these granules are carefully planted in sterile animal tissue placed at the bottom of a tube and covered with media there is evidence of increase in size of the granule but it is never marked. Immediately about the granule the long bacilli mixed with cocci may be found in large numbers. Under these conditions a very disagreeable odor is formed and the tissue becomes putrid. Here, also, it is to be noted that the spirilla forms also appear in large numbers and undoubtedly multiply under these conditions tho they have not yet been isolated pure. The odor

[1] *Jour. Infect. Dis.*, 1913, 12, p. 199.

when the entire granule is inoculated is much more intense and indeed is somewhat different in character from that produced by a pure culture of the bacillus or by the bacillus and the coccus together. The difference is therefore probably due to the presence of the spirilla forms.

In morphology the strains of bacilli when growing in pure cultures vary considerably. Fig. 7 is a photograph of a pure growth which has passed through many generations. The bacilli stain readily with carbolfuchsin, less readily with Löffler's methylene blue. Giemsa is a satisfactory stain as is also polychrome methylene blue.

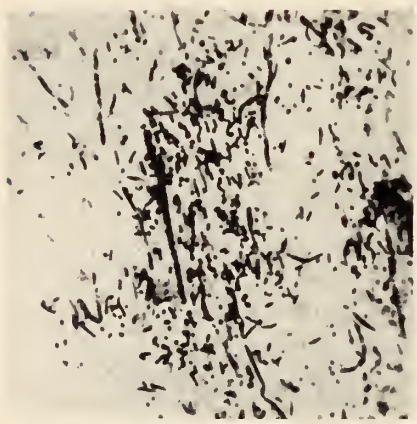

FIG. 6.—Smear preparation of a granule stained by Gram. The cocci stain clearly. The fusiform bacilli stain unevenly, some quite distinctly, others very poorly. Spirilla forms are not stained. ×1200.

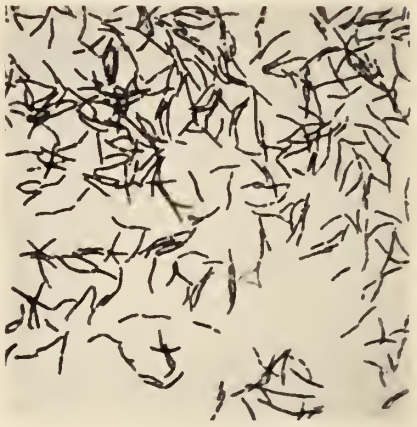

FIG. 7.—Pure culture of fusiform bacilli from actinomyces-like granule grown under anaerobic conditions on blood glucose agar. ×1200.

They are weakly gram-positive and are non-motile. Some strains have a very decided fusiform shape with pointed ends while other strains are more threadlike or filamentous in form. Often the bacilli are arranged end to end, forming at times long chains, which, however, are readily broken up into their elements. Branching never occurs. Granules within the bacilli are at times clearly visible and are best stained with methylene blue.

The relation of the bacilli and filaments is a matter which I have given careful consideration. At first I thought that they were different organisms in view of their very different morphology.

However, I am now inclined to believe that they are only different forms of the fusiform bacillus. The organisms of this type as they are obtained in cultures vary strikingly in their morphology, some being short and fusiform, others longer and more threadlike. In other respects, however, they are quite alike. They are all strictly anaerobes, their growth on media is identical and their reaction to Gram stain is alike both in the artificial cultures and also in smears made directly from the tonsil granules. I think one is therefore justified in considering the bacilli and the filaments different forms of the same organism. I do not consider this point absolutely proven, however, because one might assume that the filaments in the granules are different organisms, which do not grow in the anaerobic cultures, and that the thread forms which one does obtain in the cultures are forms of the fusiform bacilli seen in the granules.

PATHOGENICITY.

The pathogenic properties of the granule and also of the cocci and the fusiform bacilli have been studied.

A small granule was carefully washed, macerated in a suspension of salt solution, and injected into the peritoneal cavity of a half-grown rabbit. Eight days later the animal died and examination revealed this rather remarkable condition of the peritoneum. Large abscesses 1 cm. or more in diameter occurred in the mesentery and about the intestines which were more or less matted together. They were found about the spleen and also here and there adherent to the parietal peritoneum. The organs themselves were not involved and the thoracic cavity had not been invaded. The heart's blood was sterile. The abscesses had well defined connective tissue walls and the centers were soft and contained thick pus which had a foul odor. Smears of this pus made from several of the lesions always showed gram-positive diplococci and bacilli fusiform or filamentous in character. In only one smear were a few spirilla seen. The cocci and bacilli were successfully cultivated from these lesions and corresponded with the organisms found in the granules. In sections of the abscesses dense, deeply staining masses were common which on careful examination were found to consist of clusters of bacilli. With low magnification they simulated actinomyces granules. They were not the original masses injected, for these had been thoroughly macerated in a mortar. Evidently then in the tissues these bacilli grow in clusters which, however, are not as dense nor as definitely raylike as are the original tonsil granules.

A second small rabbit about six weeks old was similarly inoculated. This animal lived 21 days and became emaciated before death. Lesions similar to those found in the first animal appeared in the peritoneum. Several small nodules containing pus developed on the abdominal wall near the point of injection and the bacteriologic

examination showed the presence of cocci and fusiform bacilli as in the first instance. Several full-grown rabbits were inoculated with tonsil granules without success.

A small white rat was inoculated intraperitoneally with one of the macerated granules and died five months later. Before death it became emaciated. In the peritoneal cavity surrounded by fibrous adhesions was a large encapsulated, necrotic mass which measured 5×3 cm. and contained dirty, gray, foul-smelling pus. Smears of pus revealed cocci and threadlike bacilli but unfortunately cultures were not made. Large guinea-pigs are not susceptible, at least to the moderate-sized doses used in the above animals. Young pigs have not been tested.

The cocci, when injected in pure culture into young rabbits intraperitoneally in doses of one or two slant agar growths, will also produce abscesses similar in character to these described, but not so extensive and not as foul or putrid. When injected intravenously they may, as already stated, localize in joints causing multiple arthritis like ordinary hemolytic streptococci.

Pure cultures of the bacilli in large doses have been injected into young rabbits, rats, and guinea-pigs intravenously, intraperitoneally, and subcutaneously. In no instance have the inoculations caused lesions of any kind. By themselves, therefore, these bacilli seem to be non-pathogenic. However, when associated with cocci as in the granules or when injected in pure culture along with a pure culture of cocci, they appear to be able to grow and at least modify and perhaps intensify the pathogenic effects of the cocci.

In view of the fact that these tonsil granules have been confused by some with true actinomyces I have thought it worth while to discuss this phase of the subject somewhat in detail. First it may be well to compare the tonsil granules with actinomyces granules. The tonsil granules average larger than actinomyces granules, at times growing very much larger than the latter ever become. They are more easily decolorized by Gram stain. They have a characteristic foul odor which actinomyces never have. Branching forms do not occur and the filaments are as a rule coarser than actinomyces. The filaments are strict anaerobes; actinomyces are aerobes or facultative anaerobes. Club-shaped ends so characteristic of actinomyces do not occur. The structures mistaken by some for clubs are the brushlike forms already described. Finally when smear preparations are made, cocci,

spirilla forms, and the bacilli are always found, whereas, in true actinomyces, only the typical branching filaments appear.

The recognition of the nature of these granules in the tonsillar crypts seems to make necessary a careful revision of all that has been written on actinomycosis of the tonsils. The literature is therefore briefly summarized here.

The works of Ruge, Johne, and Jonathan Wright already referred to can be disposed of by stating that they were all certainly dealing with these mycelial granules and not with true actinomyces. In Bostroem's series[1] of 12 there are no definitely proven cases of actinomycosis of the tonsils. In only one case (Case 5) is there even a possibility of the tonsil being the atrium. He thinks the infection here probably entered through the pharynx in the immediate neighborhood of the tonsils, since the latter showed no evidence of injury. In most of his cases of actinomycosis of the lower jaw he believes that the organisms entered the tissues by means of grains through the floor of the mouth.

Thevenot,[2] under the title of "Actinomycosis of the Tonsils," reports a patient who had an attack of acute tonsillitis and who some time later developed actinomycosis on the same side of the neck. He gives no evidence, however, that makes one believe that there was any connection between the tonsillitis and the actinomycosis. No bacteriological examination of the tonsils is reported. The teeth were very bad. He refers to another case in which there was actinomycosis of the ear and near the periphery of the tonsil in the pharyngeal wall was a fistulous tract. Sections through the tonsils, however, revealed no actinomycotic lesions.

As illustrating the necessity of discussing the relation of these granules to actinomyces I would call attention to the work on actinomycosis by M. Schlegel.[3] In connection with the subject of actinomycosis of the tonsils he mentions only Ruge's findings, stating that this author found these actinomyces-like forms in the crypts. Schlegel evidently has not at all appreciated the very fundamental difference between these bodies and true actinomyces.

[1] *Beitr. z. path. Anat. u. z. allg. Path.*, 1891, 9, p. 1.

[2] *Lancette française Gazette d. hop.*, Paris, 1904, 77, p. 1070.

[3] Kolle u. Wassermann, *Handbuch d. Path. Mikroorg.*, 1912, 5, p. 329.

The above reports are all that I can find in the literature bearing on this subject. It appears that the idea is prevalent that actinomyces may enter the tissues about the neck from the tonsils but the evidence for this is not at all convincing. One difficulty in determining this point is due to the fact that the original atrium of infection is not easy to find because actinomycotic processes are slow and healing often occurs rapidly. I think there is no doubt that in some instances the infection has gained entrance somewhere in this neighborhood. Possibly it may enter at times through the tonsillar crypts but from the cases reported it does not appear that this point has yet been proven.

It should be stated that it is possible, of course, for true actinomyces to occur in tonsils in association with these raylike granules. I have carefully searched my preparations of these latter for gram-positive branching filaments typical of actinomyces but have not succeeded in finding them. In this connection I should like to refer to the work of F. T. Lord,[1] who found in smears from tonsil crypts organisms resembling actinomyces and he also observed actinomycotic abscesses in the omentum of guinea-pigs after intraperitoneal injection of the contents of tonsillar crypts. He does not carefully describe the material that he injected but merely states that it consisted of masses from the tonsils. I think there is little doubt that these granules were included. The lesions obtained by me in the omentum of rabbits contain masses of bacilli very similar to those in the omental abscesses described by Lord. However, I have never seen any structures resembling clubs in these experimental abscesses and Lord definitely describes these structures in his preparations. If these structures he describes are true actinomyces it probably means, of course, that these tonsil granules and true actinomyces may occur in the tonsillar crypts together. Without attempting to settle this point here, I will only say that, experimentally, definite abscesses can readily be produced by intraperitoneal injection of the tonsil granules and that in these abscesses structures simulating true actinomyces appear. These structures, however, are only masses of mycelium from the tonsil granules and have nothing whatever to do with actinomyces.

[1] *Jour. Am. Med. Assn.*, 1910, 55, p. 1261.

This fact must be considered in attempting to demonstrate the presence of true actinomyces in the tonsils (and also about the teeth).

Are these granules ever pathogenic for man? An answer to this question requires the discussion of a number of points. In the examination of tonsils in my own series I have never seen these mycelial masses invading the tissues. At times one sees them apparently beneath the epithelium but this is, I think, only because they have been forced there by mechanical laceration of the tonsil in its removal, the arrangement of the tonsillar tissue thereby becoming altered or distorted. It is true that others have described an invasion of the tonsillar tissue. Gappisch[1] observed this in two cases in which he states the organisms caused tissue reactions which reminded one of true actinomycosis. Wright[2] also refers to an invasion of the follicles of the tonsil by a mass of mycelium. I am not convinced that these apparent invasions were not artefacts and I believe that further data are necessary in order to establish or disprove this point.

The statement made by Wright that a marked and abnormal epithelial proliferation occurs in the tonsillar crypts about these bodies is very doubtful to say the least. I have not observed alterations of the epithelium which might not occur in a crypt with or without these granules. The granules do occur commonly in large and spacious crypts in which normally the epithelium is abundant; but this condition of the epithelium exists in all large crypts whether containing granules or not. In several tonsils I have found crypts filled with masses of these granules as large as a small pea, which have become occluded. The epithelial lining of such cavities is gray and on section the epithelial layers appear compact and more hyalinized than they do in ordinary crypts. This is due probably to pressure. I have not noted any characteristic reaction in the subepithelial tissues about such bodies that is in any way different from the plasma cell infiltration which occurs in practically all tonsils just beneath the crypt epithelium.

These bodies grow in the tonsillar crypts and from time to time are discharged. Not infrequently they become so large

[1] *Loc. cit.* [2] *Loc. cit.*

that there is difficulty in their discharge from the crypt openings where they may lie partly protruding perhaps for days. Under these conditions, as I know from my own experience, the adjacent tonsillar tissue may become irritated, slightly tender, and edematous. I do not know that this is anything more than the result of mechanical irritation but it may be due to both a mechanical and an infectious process.

The recognition that these bodies contain enormous numbers of fusiform bacilli and spirilla brings them into possible relation with fusiform infections of the tonsils, throat, and mouth, such as Vincent's angina, noma, etc. We know that these organisms are always present about the teeth and gums, especially in dirty mouths, but here in the crypts even in carefully cleaned mouths we find them often in enormous masses hidden away in a place inaccessible to ordinary cleansing.

SUMMARY.

Actinomyces-like granules are commonly found in the crypts of tonsils. They are in no way related to true actinomyces.

These granules consist of bacilli, streptococci, and spirilla. The bacilli belong to the fusiform bacillus group and have been successfully cultivated. They are strict anaerobes. They are not pathogenic for animals.

The entire granule when injected into rabbits and rats readily produces abscesses.

A symbiotic relationship probably exists between the cocci, spirilla, and bacilli.

It is very doubtful whether these granules cause hyperplasia of the epithelium of the crypts. They occur at times in cystlike spaces in the tonsil lined by compact squamous and hyalinized epithelium.

In the literature these granules have been confused with true actinomyces; misleading statements appear especially in connection with the subject of actinomycosis of the tonsils.

There does not appear to be a well authenticated case of primary actinomycosis of the tonsil yet reported.

COMPARATIVE TESTS OF SPUTUM BY THE KINYOUN AND ELLIMAN-ERLANDSEN METHODS.*

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In order to determine the comparative value of the Kinyoun and Elliman-Erlandsen methods for the examination of sputum, a series of tests was undertaken at Otisville during the summer of 1912.

Through the courtesy of the physicians at the Otisville Sanatorium, we were enabled to obtain samples of sputum from the sanatorium patients which we studied according to the method recommended by Dr. Kinyoun, comparing our results with those obtained by the sanatorium physicians in their regular routine examinations which are made by the Elliman-Erlandsen method.

The slightly modified Elliman-Erlandsen method in use at the sanatorium is as follows:

Sputum, mixed with an equal volume of $NaCO_3$ solution, is stirred and incubated for 24 hours at 37° C. From 2–3 drops of 40 per cent NaOH solution are added, and the mixture is boiled and centrifuged. As much as possible of the sediment is spread upon slides, in doubtful cases the entire sediment being examined.

By Dr. Kinyoun's method, the sputum is mixed with 2–3 volumes of sodium hypochlorite of such dilution as to contain 0.56 per cent of available chlorin. One cubic centimeter of ligroin is next added, the mixture thoroughly shaken until it has become homogeneous, then centrifuged, and an examination is made of the soapy layer lying directly below the ligroin, where the tubercle bacilli, if present, will be found to be concentrated. If the sputum is tenacious and not readily broken up, Dr. Kinyoun recommends allowing it to stand over night in the hypochlorite mixture, after preliminary shaking. The shaking is repeated next morning, after which the specimen is centrifuged and examined either with the ordinary carbolfuchsin stain or with the stronger stain recommended by Dr. Kinyoun:

4 gm. fuchsin, basic (Grubler)
8 gm. acid carbolic C. P. crystal
20 c.c. alcohol 95 per cent
100 c.c. water

Decolorization occurs with 3 per cent hydrochloric acid in 95 per cent alcohol. Methylene blue is used as a counter stain.

With the exception of the use of the centrifuge the foregoing method was followed exactly in our work. We first examined a series of specimens, centrifuging one-half and allowing the other

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to stand at room temperature, after shaking; until the next morning. It was found that the tubercle bacilli, if present, would be found gathered together in the soapy layer without previous centrifuging, and since, in some cases, our results were less good in the centrifuged portions than in those which had simply stood over night, the latter method was followed in our further work.

With nearly all specimens direct smears were made as soon as the sputum was received and these were compared with the smears from the hypochlorite and ligroin mixture.

The specimens were taken as they came, in the ordinary course of the daily routine sputum examinations, and were from cases in all stages of pulmonary tuberculosis. All precautions as to solutions, sterile glassware, and new slides were observed with both methods.

Comparison of direct with hypochlorite method.—By the direct method, 118 specimens, or 72.4 per cent, were positive; 45, or 27.6 per cent, were negative. By the hypochlorite method, 135 or 82.8 per cent, were positive; 28, or 17.2 per cent, were negative. Twenty, 12.2 per cent of the whole number and 44.4 per cent of those negative by direct examination, became positive with hypochlorite. Thirty-four, 20.8 per cent of the whole number and 28.8 per cent of those positive by direct examination, became more strongly positive with hypochlorite. Seventy-nine, 48.4 per cent of the whole number and 66.9 per cent of those positive by direct examination, showed no decided advantage with hypochlorite.

Comparison of the hypochlorite with Elliman-Erlandsen method.—The total number of specimens compared was 159. Four, or 0.25 per cent, were positive by Elliman-Erlandsen and negative by hypochlorite. Of this number, 1 specimen contained many organisms, and 3, a few organisms. Seventeen, or 10.6 per cent, were positive by hypochlorite and negative by Elliman-Erlandsen. Of this number, 7 contained many organisms; 1, a moderate number, and 12, few organisms.

CONCLUSIONS.

By the use of the hypochlorite and ligroin method, many specimens became positive which were negative by direct smear and many others showed a greatly increased number of organisms.

In the comparison of the hypochlorite and ligroin with the Elliman-Erlandsen method, allowance must be made for differences of technic between two sets of people working in different places, and a longer search may account for some of the differences in results where but few organisms were found. Leaving these cases out of account, however, it is still evident that in this series of tests, the advantage lies with the hypochlorite and not with the Elliman-Erlandsen method, since by the former, there were seven strongly positive tests and one showing a moderate number of organisms, all of which were negative by the other method, as compared with one strongly positive test by the Elliman-Erlandsen which was negative by the Kinyoun method.

TUBERCLE BACILLI IN THE BLOOD.*

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Notwithstanding the large number of investigators who have made a study of the presence of tubercle bacilli in the circulating blood, it is nevertheless a fact that the most striking point about the results so far obtained is the total lack of agreement between the reports of the different workers.

In a number of cases there is an absence of sufficient detail as to technic and controls, but leaving these records out of account there remain a long series of reports in which, after the most careful and painstaking work described in minute detail, the authors have come to absolutely contradictory conclusions.

A difference so marked could not fail to attract attention, and within the past year a number of extensive reviews of the literature have been made by different writers, especially by Bacmeister and Reuben, Elsaesser, and Rothacker and Charon.

As the majority of these articles are in German, and so far as known there is no corresponding record in English, a brief résumé of the principal articles is here given, including some of the most recent work on the subject.

The first investigator in this field seems to have been Villemin, who in 1868 obtained positive inoculation results with the blood in miliary tuberculosis. Weichselbaum, in 1884, found tubercle bacilli microscopically in otherwise sterile blood from miliary tuberculosis. Next followed Meissel, Hildebrand, Huguenin, Sticker, Gary, Bergeron, Lüdke,[1] Lustig, Rutimeyer, and others who, either microscopically or by animal inoculation, demonstrated the presence of tubercle bacilli in the blood in a varying percentage of severe cases.

As a result of this work it became recognized that bacilli could be found in the blood irregularly in the last stages of miliary tuberculosis, with a possibility that the same might be true of certain severe pulmonary infections, tho the generally accepted opinion was expressed by Bergeron in 1904 when he stated that tubercle bacilli are found in the blood in miliary tuberculosis, but only in relatively rare cases, and that in non-miliary tuberculosis, even of a rapid type, bacillemia does not exist.

A greatly added impetus was given to the investigation by the introduction of the Stäubli acetic acid method in 1908, followed by the Schnitter modification in 1909.

* Received for publication October 11, 1913.

[1] *Wien. klin. Wchnschr.*, 1906, 19, p. 949.

The Stäubli-Schnitter method is as follows: 10–15 c.c. of blood from the vein are added to a like amount of 2–3 per cent citric acid or double the amount of 3 per cent acetic acid, then carefully shaken and allowed to stand for one-half hour. The fluid is centrifuged and pipetted off, and 1 c.c. of water is added to the sediment. Then 2 to 5 times the amount of 15 per cent antiformin is added and the mixture shaken. As soon as solution is complete, which happens very quickly, the mixture is again centrifuged, the fluid pipetted off, and the slight deposit is washed and examined by the Ziehl-Nielson stain.

Schnitter[1] found acidfast bacilli in 31.6 per cent of 38 cases of tuberculosis. He found very few organisms, not more than 5 or 6 in most cases, and the majority of these were "granules." Schnitter came to the conclusion that tubercle bacilli are probably regularly present in the blood in organic tuberculosis, but that this does not necessarily lead to miliary infection, since the latter is due to a sudden explosive outburst of organisms into the blood in such numbers that the natural protective powers of the organism are unable to cope with them.

Just previous to the work of Schnitter, Rosenberger,[2] in this country, had published his report of 100 per cent positive microscopic results in 49 cases of tuberculosis of all stages, 5 miliary. Rosenberger drew blood into sterile sodium citrate, centrifuged and examined the sediment. In a later report Rosenberger brought his list of cases up to 300, in all of which he claimed to have found acidfast bacilli in the blood.

Schroeder and Cotton,[3] Burvill-Holmes,[4] Anderson, and other American workers attempted to repeat Rosenberger's work, using the same method, but utterly failed to reach the same conclusions, and a like result was reached by the English investigators, Hewat and Sutherland,[5] working with cattle, altho Forsyth[6] in England, with this method, reported 100 per cent positive results in 10 open cases of tuberculosis and negative results in 2 closed cases.

The work of Anderson[7] was especially interesting on account of his inoculation and culture tests. All direct microscopic examinations of blood were negative and of 83 guinea-pigs inoculated intraperitoneally with human blood none developed tuberculosis, but the blood of 13 tuberculous guinea-pigs gave one positive result when injected into other guinea-pigs, while of 8 infected rabbits the blood of 7 produced tuberculosis in guinea-pigs. In 3 cases rabbit-blood specimens gave cultures of tuberculosis, this being the first time, according to Anderson, that successful cultures have been reported from rabbits' blood.

With the finding by Brem,[8] and afterward by others, of acidfast bacilli in laboratory distilled water it was thought that a probable explanation could be given of the result reported by Rosenberger. Since then but little has appeared upon the subject in this country, but a large amount of work has been done in foreign laboratories, especially among the Germans.

Lippmann,[9] with the Stäubli-Schnitter method and new slides, found 11, or 44 per cent, positive in 25 pulmonary cases, all stages. He found very few bacilli and no connection between fever and positive blood test.

[1] *Deutsch. med. Wchnschr.*, 1909, 35, p. 1566. [2] *Am. Jour. Med. Sc.*, 1909, n.s., 137, p. 267.
[3] *Bull. 116, Bureau of Animal Industry, U.S. Dept. Agric.*, 1909.
[4] *Am. Jour. Med. Sc.*, 1910, 139, p. 99.
[5] *Brit. Med. Jour.*, 1909, 1, p. 1119. [6] *Ibid.*, p. 1001.
[7] *Bull. 57–59, Hyg. Lab., U.S. Pub. Health Serv.*, 1909, p. 7.
[8] *Jour. Am. Med. Assn.*, 1909, 53, p. 909. [9] *München. med. Wchnschr.*, 1909, 56, p. 2214.

Jessen and Rabinowitsch,[1] with a slightly modified Stäubli-Schnitter method, and all precautions as to slides, glassware, etc., had 10, or 28 per cent, positive results in 36 cases, different stages. Ziehl, Much, and Gram stains were used, and "granules" were included among positive results. The Ziehl stain gave best results. No agreement was evident between the clinical stage and the presence of bacilli.

Acs-Nagy,[2] with the Stäubli-Schnitter method, and careful aseptic precautions, found 11, or 45 per cent, positive blood specimens in 24 cases, of which 5 were miliary, the remainder pulmonary tuberculosis of the second and third stages. The majority of organisms were "granules." No agreement found between microscopic result and severity of case, or time before death.

Kurashige,[3] with a modified Stäubli-Schnitter method and Ziehl stain, reported positive blood findings in the astonishing ratio of 100 per cent in 155 cases, all stages. No details given as to asepsis and controls. Bacilli were present in great numbers, as many as 30 in one field. Besides the above cases of pulmonary tuberculosis, Kurashige examined the blood of 34 apparently healthy subjects and found acidfast bacilli in the blood of 20, or 59 per cent. In 3 of these cases, tuberculosis developed during the next 8 months. Guinea-pigs inoculated with microscopically positive blood from 4 apparently normal persons developed tuberculosis. In a later paper Kurashige[4] gave the results of a series of repeated examinations of blood at intervals of 1 to 2 weeks. Of the 20 cases, all stages, from whom the blood was taken, each gave positive results, and of the 114 specimens of blood, 104 were positive. None were tested in animals. Kurashige concludes that tuberculosis is primarily a bacillemia, with later localization in the tissues and the appearance of characteristic symptoms.

Sturm,[5] seeking to confirm Kurashige's findings, came to quite different conclusions. With corresponding preparations, the one stained by Much, the other by the Ziehl-Nielson method, he found by the former stain 42 per cent and by the latter only 22 per cent positive in 50 cases, all stages. Of 10 specimens from apparently normal persons, all were negative. Animal tests, however, made by the inoculation of 5–6 c.c. of blood intraperitoneally in guinea-pigs, gave the large number of 46 per cent positive results. There was no agreement between the presence of bacilli in sputum and in the blood, or between the positive blood test and the severity of the case.

Krause-Hannover,[6] using the Uhlenhuth antiformin method, had 11 per cent positive results in 32 severe cases of the second and third stages. A second series of 100 cases, all stages, gave 22 per cent positive results. Many cases positive at one time were found negative at another, and the presence of bacilli was no reliable guide for prognosis. Animal tests were not made.

Suzuki and Takaki[7] examined a great series of blood specimens and compared the results with the Pirquet test. They found acidfast bacilli by the Stäubli-Schnitter method in 98.5 per cent of 517 tuberculosis cases of all stages, and also, in 51.85 per cent of 54 apparently healthy subjects. Reagents and all materials were sterilized. Both Ziehl and Much stains were used, but the latter method was discarded as unsafe. Animal tests were apparently made, but there is only a passing allusion to positive results, no details being given.

[1] *Deutsch. med. Wchnschr.*, 1910, 36, p. 1116.
[2] *Wien. klin. Wchnschr.*, 1910, 23, p. 1313.
[3] *Ztschr. f. Tuberk.*, 1911, 17, p. 347.
[4] *Ibid.*, 1912, 18, p. 430.
[5] *Beitr. z. Klin. d. Tuberk.*, 1911, 21, p. 239.
[6] *Ztschr. f. Tuberk.*, 1911, 17, p. 436.
[7] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1911, 61, p. 149.

The writers were impressed by the agreement between the microscopic test and the skin reaction. With all persons who gave a positive skin reaction there was also a positive blood test.

Liebermeister,[1] in over 100 tuberculous cases of different stages, obtained positive inoculation results in 48 per cent. By the Stäubli-Schnitter microscopic method, with Ziehl stain and aseptic precautions, he found acidfast bacilli in each of 15 cases of open tuberculosis, and in 11 of 13 cases of closed tuberculosis. In more than 70 cases, not clinically positive, several cases of scrofula, and also in a long series of patients in various diseased conditions, not tuberculous, acidfast bacilli were found. He concludes that these conditions have some connection with tuberculosis not understood, and suggests a "secondary" tuberculosis, analogous to the secondary stage in syphilis. Liebermeister notes that when several guinea-pigs are inoculated at the same time with the same dose only one or two become tubercular. Therefore one or two negative inoculation results do not exclude tuberculosis.

Rumpf,[2] together with Zeissler, used the Schnitter method with oxalate in place of citrate, and a concentrated antiformin. All aseptic precautions were observed. He found acidfast bacilli in 100 per cent of 25 cases of tuberculosis, also in 6 former patients healed for 20 years, and in 7 healthy persons, one of whom was scrofulous as a child. Of 35 guinea-pigs inoculated intraperitoneally with microscopically positive blood, only 3 became tubercular. In 2 of these the blood came from light-closed cases, in the third from an open third-stage case.

Hilgermann and Lossen,[3] using the Stäubli-Schnitter method and all precautions with glassware and solutions, found acidfast bacilli in 17, or 25 per cent, of 64 lung cases, all stages, but made no animal tests. Authors found no connection between positive blood and temperature conditions. The course of the disease was less favorable in the majority of the cases with positive blood, but this could not be attributed to a beginning miliary process since in many cases the positive blood test was followed by a temporary improvement.

Kennerknecht[4] examined blood in 120 children, tuberculous, tuberculosis suspects, and apparently healthy. Using the Stäubli-Schnitter technic with the addition of absolute alcohol, the Ziehl and Gram stains, new slides, and all precautions with glassware and reagents, acidfast bacilli were found in 109, or 91 per cent, of all specimens. Very few bacilli, sometimes more in the beginning or healing stage than at the height of disease, and "granules" included in positive findings. Of 13 guinea-pigs inoculated intraperitoneally all are said to have become tuberculous, but doubt is thrown upon the autopsy results by Bacmeister and also by Rabinowitsch.

Klemperer,[5] with the Stäubli-Schnitter method and Ziehl stain, examined blood in 39 cases of tuberculosis, suspected tuberculosis, other diseases, and healthy persons. He found acidfast bacilli in the majority of tuberculosis cases and tuberculosis suspects and in one case of liver cirrhosis, but never in healthy persons. Only characteristic well-stained bacilli counted. These were generally few in number. Full details of aseptic precautions with solutions and glassware. No animal tests. Klemperer, observing that blood free from bacilli dissolves in antiformin more slowly than blood in which bacilli are found, argues from this a probable lessened resistance. In

[1] *Med. Klin.*, 1912, 8, p. 1018.

[2] *München. med. Wchnschr.*, 1912, 59, p. 1951.

[3] *Deutsch. med. Wchnschr.*, 1912, 38, p. 895.

[4] *Beitr. z. Klin. d. Tuberk.*, 1912, 23, p. 265.

[5] *Therap. d. Gegenw.*, 1912, 53, p. 433.

opposition to Suzuki and Takaki positive blood results were not found in all cases giving positive Pirquet reaction. Klemperer thinks the finding of bacilli of worth for diagnosis, but not for prognosis.

Ranström-Upsala[1] used the method of Rosenberger and the Uhlenluth antiformin method, sterile glassware, and all aseptic precautions. Of 36 cases of lung tuberculosis, 0, or 25 per cent, showed acidfast bacilli. This author alone seems to have found a definite ratio between temperature and positive blood test.

Bacmeister and Reuben,[2] with the Stäubli-Schnitter method, sterile glassware, and filtered fluids, examined blood from "a great series" of cases of lung tuberculosis in the third stage, from 15 cases in the first stage, from 8 non-tuberculous subjects, and from a number of sound rabbits. In all of these specimens acidfast bacilli were found. Granules were not counted. Blood from 15 light and severe cases was inoculated into rabbits and in part into guinea-pigs. These tests gave only negative results. The authors came to the conclusion that the chemical reaction of acetic acid and antiformin in the Stäubli-Schnitter method may give rise to appearances which simulate tubercle bacilli.

Lydia Rabinowitsch[3] criticized the use of rabbits by Bacmeister instead of guinea-pigs which she found much more suitable for the study of human tubercle bacilli. She also questioned the finding of bacilli in normal rabbits by Bacmeister and by Sturm. Rabinowitsch made the interesting observation that the blood of highly tuberculous animals frequently fails to produce tuberculosis when injected into guinea-pigs, but that after the tuberculous animals have been injected with tuberculin the blood then gives a positive inoculation result. She quotes the experience of Suzuki and Takaki in this connection, and also the observations of Virchow and Orth published 22 years before, and agrees with the latter authors that the above result is probably due to the "mobilizing" of the bacilli through the action of the tuberculin.

On the above point Bacmeister[4] in a new series of tests comes to the same conclusion as Rabinowitsch. In 15 cases of light tuberculosis he found acidfast bacilli, but with all of these specimens animal tests were negative. Upon injecting the patients with tuberculin and taking blood at the height of the reaction he was able to produce typical tuberculosis in 4, or 13.3 per cent, of 30 inoculated animals. The acidfast bacilli found in his earlier work with rabbits and guinea-pigs he later considered to have been "Futterungs" bacilli.

Rosenberg[5] examined blood in a series of surgical as well as pulmonary cases, also in healthy persons. In the healthy he found nothing in any case, but in his other tests acidfast bacilli were found in nearly every specimen. Very few organisms were present in any case and these found after long search. There was no definite relation between severity of disease and bacilli in blood, and the latter condition is considered of no value for prognosis, tho thought to be specific as a means of diagnosis.

Nobécourt and Darré[6] studied the question of bacillemia in tuberculous children of various ages with different forms of tuberculosis, using exclusively the method of guinea-pig inoculation. Ten to 20 c.c. of blood were divided into equal parts and inoculated intraperitoneally into 2 or 3 guinea-pigs.

[1] *Deutsch. med. Wchnschr.*, 1912, 38, p. 1535.

[2] *Ibid.*, p. 2350.

[3] *Berl. klin. Wchnschr.*, 1913, 50, p. 110.

[4] *München. med. Wchnschr.*, 1913, 60, p. 343.

[5] *Ibid.*, p. 404.

[6] *Bull. Soc. d'étud. scient. sur la Tuberc.*, 1912, 2, p. 176.

Of 40 patients, in acute, subacute, and chronic stages, only 4 gave positive results. The first, a child of 4 years, showed a clinical picture of general tuberculosis. A guinea-pig, inoculated intraperitoneally, died 12 days later and showed fresh granulations in the spleen containing acidfast bacilli. A second animal inoculated with a fragment of this spleen developed general tuberculosis. Another specimen of blood taken 8 days later, while the fever was still high, gave only negative results in 2 guinea-pigs, 1 inoculated subcutaneously, the other intraperitoneally. This patient was later discharged, apparently completely cured, and the diagnosis would have remained in doubt but for the positive animal test.

The second positive case was a little girl of 4 years with general tuberculosis of an extremely rapid type, the pulmonary symptoms predominating. Two guinea-pigs inoculated intraperitoneally developed tuberculosis.

The third positive blood case was in a boy of 9, with typical tuberculous meningitis. Blood taken 3 days after beginning of disease was negative in 2 pigs, but a second specimen taken 6 days later produced general tuberculosis by intraperitoneal inoculation.

The fourth positive blood was from an infant with acute tuberculous bronchopneumonia. Two inoculated guinea-pigs developed general tuberculosis.

The authors point out the opposite inoculation results with different blood specimens from the same patient, and they emphasize the importance of the period of the disease when the specimen is taken, considering this a more essential point than the quantity of blood inoculated.

Querner[1] used only animal tests. He injected guinea-pigs intraperitoneally with sediment obtained by the Stäubli-Schnitter method from the blood of 37 chronic pulmonary cases in all stages. In 3 cases two inoculations were made, making 40 tests in all. The animals, killed 27–143 days after inoculation, proved to be negative in every instance. The authors made control tests of the action of antiformin on tubercle bacilli, and found no lessening of virulence produced.

Krabbel[2] made microscopic examinations of blood in 35 cases of tuberculosis of lung, third stage, tuberculosis of bones, of glands, or of skin. Using Stäubli-Schnitter method, new slides, sterile glassware, and stains constantly renewed, he found acidfast bacilli in 20, or 57.1 per cent. No animal tests made. Krabbel finds no significance in positive blood tests as they may be negative in advanced cases, and positive in cases getting well.

Brandes and Mau[3] confined their investigations to surgical cases. With the Stäubli-Schnitter method and Ziehl stains, they examined blood in 40 cases of surgical tuberculosis of all stages (height and beginning of disease, and clinically healed). They used sterile glassware, new slides, and water freshly distilled, and found acidfast bacilli in 18, or 45 per cent. Bacilli in blood were thought to indicate tuberculosis, but the test was not considered of practical value.

De Verbizier[4] examined blood in 15 cases of tuberculosis by the Stäubli-Schnitter and Rosenberger methods, but found no acidfast bacilli in any case. Fifteen guinea-pigs inoculated intraperitoneally were all negative. The author concludes that bacillemia must be very rare in tuberculosis.

Rogers and Murphy,[5] using the Stäubli-Schnitter and absolute alcohol method,

[1] *München. med. Wchnschr.*, 1913, 60, p. 401.

[2] *Deutsch. Ztschr. f. Chir.*, 1913, 120, p. 370.

[3] *Deutsch. mil. Wchnschr.*, 1913, 39, p. 1137.

[4] *Rev. de mél.*, 1913, 33, p. 161.

[5] *Jour. Am. Med. Assn.*, 1913, 60, p. 995.

tested blood in 50 cases of pulmonary tuberculosis, all stages, and in 5 apparently normal persons. They found acidfast bacilli in all, morphologically identical with tubercle bacilli. Authors made numerous control tests. The water used was tested by adding albumen, centrifuging one-half to one hour, and examining the sediment, with results uniformly negative. A small loopful of tuberculous culture was then added to 1 c.c. of water and a little albumen. After the mixture was carried through the same process as test specimens, the tubercle bacilli appeared on the slides in clumps. To eliminate crystals the authors made a series of tests with combinations of acetic acid, alcohol and albumen, acetic acid and solutions of alkalies and antiformin, but none of these tests gave crystals at all like the appearances found in the blood. These results are of interest as they are in opposition to the suggestion of Bacmeister and Reuben.

Dreesen[1] tested blood in 70 cases of tuberculosis, tuberculosis suspects, and apparently normal persons. Blood was drawn into freshly distilled water, kept in the dark until next day, and then centrifuged for 1 hour. One-half of the sediment was treated with antiformin for 2–3 minutes, diluted with freshly distilled water, centrifuged, and sediment examined with Ziehl stain. Only well-stained rods counted. Acidfast rods and granules were found in 74 per cent of the surely tubercular, 55 per cent of the suspicious cases, and 46 per cent of the apparently normal cases. In marked contrast with these findings was the result of animal tests. The other half of the blood sediment described above was emulsified with 2 c.c. of sodium chlorid and injected into 2 guinea-pigs, one pig being inoculated in the groin, the other in the peritoneum or in the liver. Of 128 pigs thus treated 39 died prematurely, the other 89 lived 7–8 weeks, and only one of all these animals gave any evidence of tuberculosis. This pig, inoculated from a case of advanced phthisis, showed true tuberculous lesions, and tubercle bacilli in smears. Tuberculin tests of all the other animals were negative.

Fränkel[2] made examinations in 25 cases including tuberculosis second and third stages, catarrh, and other conditions. Eighteen specimens of blood were examined microscopically by the Stäubli-Schnitter method, Ziehl, Much, and Spengler stains. Four of these specimens showed acidfast bacilli. Forty-two guinea-pigs were inoculated subcutaneously and intraperitoneally and of these only 2 developed tuberculosis. The blood injected in 1 pig was from a case of advanced phthisis, but in the other animal the blood was from a case of chronic bronchitis with no evidence of tuberculosis, and with blood and sputum negative microscopically.

Bernard, Debré, and Baron,[3] after trying various methods, selected the following for their work. Ten cubic centimeters of blood were taken from the vein into a sterile tube containing 20 c.c. of 30 per cent alcohol. Complete laking was obtained by the gradual addition of about 30 c.c. of 40 per cent alcohol, with vigorous shaking. After centrifuging half an hour, the fluid was pipetted off. The sediment was redissolved in 40 c.c. of 40 per cent alcohol, shaken energetically, and 1–2 drops of 10 per cent alcoholic solution of soda added. The fluid thus obtained is clear and but slightly viscous, if an excess of soda is avoided. The deposit obtained by centrifuging is then examined by the Ziehl stain.

The authors claim many advantages for this as compared with other methods. They examined 41 specimens of blood from 36 tuberculous patients in all stages.

[1] *Med. Klin.*, 1913, 9, p. 580.

[2] *Deutsch. med. Wchnschr.*, 1913, 39, p. 737.

[3] *Bull. Soc. d'étud. scient. sur la Tuberc.*, 1912, 2, p. 154.

Thirty-seven specimens were negative. In 4 cases the results were completely or partially positive. In the first case the blood was taken from a patient with tuberculous meningitis 3 days before death. Three apparently typical bacilli were found. Of 2 guinea-pigs inoculated, 1 was negative 3 months later; the second developed tuberculous lesions containing acidfast bacilli. Another pig inoculated with gland material from this animal died prematurely. The second positive blood specimen was from a case of miliary tuberculosis and showed a mass of apparently typical bacilli. Guinea-pigs inoculated died accidentally. The third specimen was from a case of pulmonary tuberculosis with cavities. Acidfast bacilli were found on the accidental death of an inoculated animal. In the fourth case the blood was from a patient with miliary tuberculosis, and the inoculated guinea-pig developed typical lesions.

The authors question the accuracy of the methods usually employed, and especially the use of antiformin, as they have found acidfast granules in the deposit at the bottom of a bottle of antiformin, and they suggest the possibility that acidfast organisms may vegetate in this fluid.

Bernard, Debré, and Baron[1] made a second series of experiments with animals experimentally infected. They selected the guinea-pig because it was possible to inject the entire blood of an animal into another. An inoculation dose was prepared by grinding up one centigram of a glycerin potato culture of tubercle bacilli in a sterile mortar, adding 10 c.c. of sterile salt solution, and shaking into a homogeneous emulsion. Of this emulsion 1 c.c., equaling 1 milligram of culture, was then injected into the external jugular vein of guinea-pigs. Great care was used to avoid infecting the surrounding tissues, and the most careful technic was employed in the second part of the experiment when the blood was recovered from the carotid artery and introduced by transfusion into other guinea-pigs. Of 23 guinea-pigs injected with the emulsion all sickened very quickly and severely, and showed the lesions of general miliary infection. The blood of these pigs, drawn at intervals of from 20 minutes to 29 days, produced tuberculosis in every instance when introduced into other guinea-pigs, and the microscopic blood examination was also positive in every case. Inoculations were also made of the urine and bile which had been collected with every aseptic precaution from the infected pigs. Leaving out the animals which died prematurely, all the urine inoculations were positive (pigs inoculated with urines obtained 2, 8, 10, 16, 21, 22, and 29 days after injection), and 5 out of 8 inoculations of bile were positive (bile obtained 2, 16, 20, 21, and 29 days after injection).

In 15 guinea-pigs injected with the same dose of tubercle bacilli subcutaneously the blood was found to be much less virulent, 5 out of 16 guinea-pigs being positive with blood drawn at the seventh hour, 12, 18, 22, and 31 days later. Authors conclude that after intravenous inoculation with tubercle bacilli the organisms remain constantly present in the blood until the death of the animal. The same is true of the urine, probably, but not proven with regard to the bile. Bacillemia inconstant after subcutaneous injection.

Rist, Armand-Delille, and Lévy-Bruhl[2] question the results obtained by Bernard, Debré, and Baron. Authors made tests in 50 cases using the Stäubli-Schnitter method with Kurashiga modification, 1 c.c. of blood examined microscopically by Ziehl and Much stains, and 9 c.c. injected intraperitoneally into guinea-pigs. Of 50 cases, 40

[1] *Bull. Soc. d'étud. scient. sur la Tuberc.*, 1913, 3, p. 52.

[2] *Ibid.*, p. 19.

gave a negative result by Ziehl and by Much, and 7 were positive sometimes by Ziehl and sometimes by Much. Guinea-pigs inoculated from these 7 cases were all negative. Three specimens were positive both by Ziehl and by Much. In the first case the blood was from a patient with a cavity. Guinea-pig killed in 2 months showed no sign of tuberculosis. In the second case, the guinea-pig showed apparent lesions of peritoneal tuberculosis and granulations in liver and spleen, but a second guinea-pig inoculated with this material remained healthy. In the third case, the guinea-pig showed lesions of tuberculosis, and a few doubtful bacilli were found in smears. Authors point out the contrast between the work of Bernard, Debré, and Baron, and their own results in which they have but one positive case among 50 tested. They are willing to admit a more or less rapid and transitory dissemination of the bacilli in certain cases, but consider this due to a condition of allergic sensibility. This condition, common to all the adult tuberculous, is incompatible with a septicemia. The extraordinary percentage of bacilli in blood reported by some is thought by the authors to be due to technical errors.

During the last few months there have been three important contributions to this subject in articles by Elsaesser, Rothacker and Charon, Lange and Lindemann.

Elsaesser,[1] after an extensive review of the literature, gives the results of his own tests. He examined blood in 41 cases of pulmonary tuberculosis, generally of a severe type, the blood being obtained not long before death. Many different methods and stains were employed, but acidfast bacilli were found in only 3 cases, and in these the Zeissler modification of Schnitter's method was used together with Ziehl's stain. In 25 cases guinea-pigs were inoculated with 1 c.c. of blood, 2 animals being used, one injected intraperitoneally, the other subcutaneously. Each pig was examined as it died. If both of a pair remained alive for some months one was killed and examined. In all cases these tests gave negative results.

Elsaesser calls attention to the strongly contrasting results of different workers, and also to the general discrepancy between the microscopic findings and the results of animal tests. He warns against conclusions based upon the microscope alone since the presence of tubercle bacilli in the blood is not proven without successful animal inoculation. At the same time, even with true bacilli, this proof may not always be obtainable, since if the bacilli should be present in very small numbers the organism might be able successfully to cope with them.

Rothacker and Charon[2] give a synopsis of the investigations of many authors as to the question of bacillemia in the tuberculous, and report upon their own work in the examination of 46 blood specimens from tuberculous patients in all stages. They used the Stäubli-Schnitter method and Ziehl stain, with every safeguard as to glassware, instruments, solutions, etc. As an additional precaution the smears were not dried with filter paper, and many controls of all materials were made. In particular, control tests were made, suggested by the work of Kahn with blood corpuscles, Kahn having stated that the stroma of red blood corpuscles, owing to their content of cholesterin and lecithin, were more or less acidfast in nature, and thus might give rise to confusion with tubercle bacilli. The authors confirmed Kahn's statement as to the acidfast property of lecithin and cholesterin, but found this to be weak in quality, and did not agree with Kahn as to the possibility of confusion with tubercle bacilli. Of the 46 blood specimens examined all specimens from patients in the first

[1] *Beitr. z. Klin. d. Tuberk.*, 1913, 26, p. 367.

[2] *Centralbl. f. Bakteriol.*, I. *Orig.*, 1913, 69, p. 478.

stage were negative; about 13 per cent of those from the second stage, and 60 per cent of those from the third stage were found to contain acidfast bacilli. Forty-six guinea-pigs were inoculated, but only one developed tuberculosis, this pig having been injected with blood from miliary tuberculosis. The authors conclude that the results of the Stäubli-Schnitter method are unreliable, and that animal inoculation is the only safe test for the blood.

Lange and Lindemann,[1] in a paper read before the recent meeting of the Freien Vereinigung für Microbiologie in Berlin, gave the results of their tests upon blood specimens from 78 cases of pulmonary tuberculosis in all stages, 2 tuberculosis suspects, and 2 healthy persons. The authors were able to report only on the microscopic work as the animal tests, begun some months before, were still in progress. Zeissler's modification of Schnitter's method was used, together with Ziehl's stain, and full details are given of the most extreme care with glassware, solutions, instruments, etc. A thorough search was made for one to one and one-half hours on each slide, in over one-third of the specimens both investigators working together, and controlling each other, but all examinations were absolutely negative. Not only were there no acidfast bacilli found, but no definite bacilli of any sort.

In contrast to Bacmeister's statement the blood of 4 normal rabbits, examined in the same manner, also gave entirely negative results.

As a control the authors made suspensions of tubercle bacilli in varying degrees of dilution in guinea-pig blood, then centrifuged and found that in the dilution of 1 to 100 millions the bacilli were still to be found microscopically. In the dilution of 1 to 500 millions they were no longer detected. This "negative" sediment was injected into guinea-pigs, still living at the time of report.

Of the 250 guinea-pigs inoculated in the original blood tests 38 had died, but only one of these had given any evidence of tuberculosis, and in this animal a careful search failed to show tubercle bacilli or any acidfast appearances. The authors conclude that the many reports of positive microscopic results are to be received with much skepticism.

An interesting discussion with opinions both in support and in opposition followed the above paper.

EXPERIMENTS.

Through the courtesy of Dr. Wilson, and the physicians stationed at North Brother's Island, specimens of blood were obtained with Miss Smeaton's assistance from patients in the second and third stages of tuberculosis, all giving a history of tubercle bacilli in the sputum.

A recent study of tuberculous sputa with Miss Smeaton by the Kinyoun ligroin and sodium hypochlorite method as compared with the Elliman-Erlandsen method had shown the former to be an excellent method for the detection of tubercle bacilli in sputum, and as a series of control tests had proved it to be of equal value for

[1] *Centralbl. f. Bakteriol., Ref.*, 1913, 57, Beiheft, p. 285.

the finding of tubercle bacilli in blood specimens, this was the method chosen for the present work.

The technic pursued was as follows: with aseptic precautions from 3–5 c.c. of blood were drawn from the vein at the elbow into a sterile tube containing 2 c.c. of 10 per cent sodium citrate solution. To the blood and sodium citrate in the test tubes was added about three times the amount of sodium hypochlorite of such dilution as to contain 0.56 per cent of available chlorin. One cubic centimeter of ligroin was next added, the mixture thoroughly shaken and allowed to stand over night at room temperature, when examination was made of the lower surface of the sharply separated layer of ligroin. The centrifuge was not used, as originally recommended by Dr. Kinyoun, for in our work with sputum we had demonstrated, by numerous control tests, that if tubercle bacilli were present in the specimen they would be found without previous centrifuging in the soap-like stratum on the lower side of the ligroin layer, and in some cases our results had been found to be less favorable with the centrifuged portion of a specimen than with that portion which had simply stood over night after shaking.

New slides, which had been carefully prepared by boiling in soda solution, rinsing in water, rubbing with alcohol, and thoroughly firing in the flame, were covered with as much as possible of the material to be examined, the smears dried in the air, fixed by moderate heat, and stained with the strong solution of carbol fuchsin recommended by Dr. Kinyoun:

4 gms. fuchsin, basic (Grubler)
8 gms. acid carbolic C.P. crystal
20 c.c. alcohol 95 per cent
100 c.c. water

Decolorization was obtained with 3 per cent hydrochloric acid in 95 per cent alcohol. Methylene blue was used as a counter stain.

All glassware was specially cleaned and sterilized and all solutions of every kind coming in contact with the specimens were passed through the Berkefeld filter until it became evident that the latter precaution was not necessary when unfiltered fluids were used as with the customary routine staining for tubercle bacilli.

Control tests were made during the entire course of the work by means of tubercle bacilli from pure cultures suspended in sterile

salt solution. When dilute suspensions of these organisms were added in very small quantities to tubes of sterile blood together with ligroin and hypophosphite, and the further process carried out exactly as with the test specimens, the bacilli were invariably found to be sharply localized beneath the ligroin layer, and could be readily demonstrated in smears even after several days in the hypochlorite mixture.

In all, blood specimens were taken from 50 tuberculous patients, two specimens obtained from one individual, one month apart, making a total of 51 specimens examined. Of these, 13 were from patients in the second stage, and 38 from patients in the third stage of the disease, 4 of the latter being bed patients. Six patients have since died, the first 3 at intervals of from 17 days to 1 month, the last 3 in from 1 month to 5 weeks after the taking of the blood specimens.

The most careful search was made for tubercle bacilli, from 150 to over 300 microscopic fields being examined with each specimen, but in all cases the results were absolutely negative, no acidfast organisms of any kind being found in the smears. A few rods were occasionally seen, but never one which showed acidfast staining, notwithstanding the precaution of having at least one smear from each specimen overstained and only so slightly decolorized that even partially acidfast organisms must have been detected if present.

Owing to the invariably negative microscopic results no animals were inoculated, as it had been thought best to wait for some positive indications from the microscopic work before beginning animal tests.

As a preliminary to this work, examinations of blood taken from normal animals and human beings were made by the same method. In this way specimens from 13 horses, 9 goats, and 3 laboratory workers were examined with negative findings in all cases.

To summarize, the results of the present work are as follows: a total of 51 specimens from the lung were all negative. Of these, 38 specimens were from the third stage and 13 from the second stage. All the specimens from normal controls, including 13 horses, 9 goats, and 3 humans, were negative.

CONCLUSIONS.

The entirely negative results obtained during the present work by the microscopic examination of 51 specimens of blood taken from patients in advanced stages of tuberculosis are in agreement with the experience of a large number of observers. In other cases acidfast bacilli have apparently been found in the blood of tuberculosis patients by different investigators, but such bacilli have been reported present in normal as well as tuberculous individuals. Since organisms morphologically identified with tubercle bacilli are present occasionally in water and elsewhere it is fair to assume that some, at least, of the acidfast bacilli observed in blood were not tubercle bacilli, and where there are no confirmatory animal tests the proportion of specimens containing true tubercle bacilli must remain an open question.

Not only are animal tests indispensable in all cases before positive microscopic results can be accepted, but it is evident that in all microscopic work the utmost care must be exercised as to filtration of solutions and sterilizing of glassware; also care must be taken that no acidfast organisms attach themselves to the slides during the various processes of fixing and staining.

Tho the animal tests included in the above summary of the work of different investigations are characterized by a striking lack of agreement, in some instances according to the testimony of well-known observers, the results indicate that bacillemia is occasionally present in tuberculosis in a wider range of cases than was formerly supposed. In view of the findings of Sturm, Rumpf, Liebermeister and others it would seem that there must be some modification of the generally accepted opinion that tubercle bacilli are to be found in the blood stream only in cases of miliary tuberculosis. On the other hand results such as these stand in need of much confirmatory evidence since in the great majority of cases no evidence of tuberculosis has been found after the inoculation of animals with the blood of tuberculous patients.

In all, abstracts are here given of 38 investigations exclusive of the present experiments. In 15 cases only microscopic results were reported, no animal tests being made. In 7 series of tests the

results of animal inoculation were entirely negative. In 9 series the majority of the inoculation results were negative. In 2 reports all inoculation results are given as positive. In 3 reports a large percentage of inoculation tests was positive, while in 2 papers positive inoculation results are reported without convincing details.

In view of the mass of contradictory evidence it is important that all possible proof including cultural tests with material from the infected animal should be given in all cases of successful animal inoculation. At the same time, the experience of Liebermeister and others as to the irregular action of the same blood upon different animals must not be forgotten since one or two negative inoculation results may not be sufficient proof that no bacilli are present in a given specimen.

Judging from the negative microscopic results in the present series of specimens from so many severe cases, it must be concluded that acidfast bacilli are at least infrequent in the blood of tuberculous individuals, and while the nature of the organisms reported is a question of much interest, yet it is not a point which seems likely to prove of value in the diagnosis or prognosis of the disease.

MULTIPLE ARTHRITIS DUE TO A FRIEDLÄNDER BACILLUS.*

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There seems but little doubt that many cases of chronic multiple arthritis are caused by streptococci and closely related bacteria. The localization of these organisms in the joints when injected into the circulation of experimental animals has been frequently observed. The pathological lesions attending such localization are discussed in the recent articles of Koch[1] and Jackson.[2] Concerning organisms other than streptococci as a cause of chronic multiple arthritis but little is to be found in the literature. The organism concerned in this report was isolated from the following case:

Swedish woman, 25 years old, a few years after coming to America, began to have trouble with the joints of her hands. The joints grew stiff and painful and became enlarged. The disease began insidiously, progressed slowly, and later affected the joints of the toes of both feet, the wrists, the right elbow, and to a slight extent the ankle and shoulder joints. At the time the patient was first seen, the disease had existed about 7 years. The joints most markedly affected were the carpal. These were distended with a fluid of a mucoid consistency, which contained many leukocytes mostly of the round cell type. The fluid was repeatedly found sterile by various cultural methods. The cartilages and the ends of the bones, as shown in the accompanying skiagraph, presented extensive atrophy and some exostosis.

On account of a history of chronic tonsillitis, cultures were made from the tonsils on blood agar plates. Colonies of *B. mucosus* (Friedländer bacillus) outnumbered all the other colonies on repeated examinations. Several varieties of streptococci were isolated and tested on rabbits in varying doses without producing joint lesions. When injected intravenously the Friedländer bacillus, on the other hand, produced joint lesions in rabbits and dogs in every instance. The organism was very toxic for rabbits and one-fourth of a 24-hour agar slant killed a large rabbit in 12–24 hours. In 6 rabbits receiving from one-tenth to one-fifth of 24-hour cultures death occurred in from 24–72 hours and in every case the bacilli were found in smear and cultures from the slight exudate in the joints of the animals, purulent pericarditis was found with bacilli present in the pus.

* Received for publication December 9, 1913.

[1] *Ztschr. f. Hyg. u. Infectionskrankh.*, 1912, 72, p. 321.

[2] *Jour. Infect. Dis.*, 1913, 12, p. 364.

In order to produce more marked joint changes 4 rabbits were given repeated intravenous injections beginning with one-fortieth of a 24-hour culture and doubling the dose every few days until one-half of a 24-hour culture was given at a dose. In this way extensive changes in the joint structure with exudation, atrophy, and bone proliferation were produced. Fig. 2 illustrates the changes. In one of the animals,

FIG. 1.—Skiagraph of hands showing destruction of cartilage, absorption of bone and, in places, exostoses.

killed 12 days after the last injection, the exudate into the joints was sterile. In the more chronic cases the leukocytes present in the exudate were mostly of the round cell type, while those of the acute cases were of the polymorphonuclear type. The joints affected most markedly were the carpal and phalangeal joints especially of the forelegs.

The bacillus with which these changes were produced is a gram-negative encapsulated organism, occurring singly and in

pairs, and forming a heavy transparent slimy growth. A comparison was made with bacilli of similar morphology from other sources. Three other organisms were used, one isolated from a pneumonia lung, one from an acute rhinitis, and one from a chronic pharyngitis. None of these organisms produced joint lesions in

FIG. 2.—Microscopic appearances in chronic arthritis produced in a rabbit. Note exudation, consisting mostly of round cells, and destruction of cartilage and bone with exostosis.

animals and all were much less toxic. Differences in the powers of fermentation of these organisms are shown in Table 1.

Perkins[1] classifies bacilli of the Friedländer type as follows: I. The aerogenes capsulatus type ferments all sugars with gas.

[1] *Jour. Infect. Dis.*, 1904, 1, p. 241.

II. The *B. pneumoniae* type, ferments all sugars excepting lactose with gas. III. *B. lactis aerogenes* ferments all sugars excepting saccharose. According to this division the bacillus causing arthritis is of type I while the other organisms studied are of type II.

TABLE 1.

MEDIA	ARTHRITIS		PHARYNGITIS		RHINITIS		PNEUMONIA	
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
Dextrose	Acid-gas	Acid-gas	Acid-gas	Acid-gas	Acid-gas	.....	Acid-gas	Acid-gas
Mannite	" "	" "	Acid	Acid	Acid	.....	.....	.....
Milk Lit.	Acid-coag.	Decol.-coag.	.....	Slight acid; no coag.	Decol.; no coag.	Decol. coag.	.....	Slight acid; no coag.
Lactose	Acid-gas	Acid-gas	.....	.....	.....	Acid	.....	.....
Inulin	.....	.....	.....	.....	.....	Acid	.....	.....
Gelatin	.....	.....	.....	.....	.....	.....	.....	.....

Following a series of injections of killed bacilli increasing in dosage from one million to one billion organisms a marked improvement occurred in the patient, followed, however, by a relapse accompanying an acute bronchitis, tonsillitis, and rhinitis. Following this, further vaccine treatment produced but little effect until the removal of the tonsils after which vaccines were again given with improvement and absorption of exudation which has lasted for about six months.

OBSERVATIONS ON A SPONTANEOUS TYPHOID-LIKE EPIDEMIC AMONG WHITE RATS.*

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I. INTRODUCTION.

During the past winter, many of the albino rats used in this laboratory died of an acute infectious disease. Among the rats which became infected were five litters, the majority of which had been thymectomized when from 10 days to 2 weeks old, and which had been kept under close observation for several months. As these rats showed symptoms of illness, they were killed together with control, unoperated animals of the same litters, and a routine histological examination made of the principal organs. The lesions found proved to be so striking and to resemble in so many respects the lesions of human typhoid fever, that it seemed desirable to isolate the infecting organism and to study the disease experimentally.[1]

Interest in epidemics among rats and mice dates back to the first publication of Löffler[2] in 1892. Löffler isolated from spontaneously infected laboratory mice a motile, gram-negative bacillus of the typhoid-colon group to which he gave the name *B. typhi murium*. This bacillus was pathogenic for field mice, but not regularly so for house mice; grey rats could not be infected. The following year Löffler used cultures of this organism to combat a plague of field mice which were ravaging the crops in

* Received for publication August 19, 1913.

[1] A preliminary report of our work was presented at the meeting of the New York Pathological Society in May.

[2] *Centralbl. f. Bakteriol.*, 1892, 11, p. 129; 1893, 13, p. 647.

Thessaly, and his successful experiences inaugurated the use of bacteria of this group for the wholesale destruction of rodents.

Danysz[1] in 1900 isolated from an epidemic of field mice in Charny-en-Seine a similar gram-negative bacillus which at first exhibited marked virulence for rats. The Danysz virus has been repeatedly studied, both as to its bacteriological relationships and as to its practical applicability to the destruction of rats.

Issatschenko,[2] Tartakowsky,[3] Schilling,[4] Trautmann,[5] and Schern[6] have described spontaneous epidemics among laboratory rats, in all of which bacilli of the typhoid-colon group have been isolated. There is further an abundant literature dealing with the interrelationships of the different strains of rat and mouse viruses, and their identity with one or another type of paratyphoid, enteritis, or hog cholera bacillus. We may refer here to the papers of Bahr,[7] Raebiger and Grosse,[8] Bainbridge,[9] Rosenow,[10] Kutscher and Meinicke,[11] Xylander,[12] Mereschowsky,[13] Bongert,[14] and Steffenhagen,[15] and shall have occasion to cite some of these workers in discussing the identity of the bacillus isolated by us.

Interest in the study of these organisms has thus centered largely upon their practical use in the extermination of rats and mice, and to this end cultures prepared by various hygienic institutes and commercial firms have been used on a large scale. Furthermore, the close relationship of the "Rattenschadlinge" to paratyphus and meat-poisoning bacilli, has suggested that their indiscriminate use might give rise to human infections, and this possibility appears to have been established by the experiences of Handson, Williams, and Klein,[16] Mayer,[17] Trommsdorf,[18] Shibayama[19] and Fleischanderl.[20]

In contrast to the abundant literature dealing with the bacteriologic and hygienic aspects of the disease are the relatively scant references to the pathological features. The gross changes, it is true, have been repeatedly described, and with considerable uniformity save as regards the intestinal lesions. On the other hand, so far as we are aware, a careful histological study has not been published. Mallory and Ordway in 1909[21] first called attention to the resemblance of the lesions produced by the injection of the Danysz bacillus in rats to the lesions of human typhoid, and demonstrated illustrative preparations at the meeting of the American Association of Pathologists and Bacteriologists. Beyond a brief abstract, no complete report of their work has appeared. During the progress of our study, there appeared a paper by Ordway, Kellert, and

[1] *Ann. d. l'Inst. Pasteur*, 1900, 14, p. 193.
[2] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1898, 23, p. 873; 1902, 31, p. 26.
[3] Ref. in *Baumgarten's Jahresber.*, 1902, 18, p. 597.
[4] *Arb. a. d. k. Gsndhtsamte*, 1902, 18, p. 108.
[5] *Ztschr. f. Hyg.*, 1906, 54, p. 104.
[6] *Arb. a. d. k. Gsndhtsamte*, 1909, 30, p. 575.
[7] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1905, 39, p. 263.
[8] *Ibid.*, 1910, 54, p. 231.
[9] *Jour. Path. and Bacteriol.*, 1909, 13, p. 443.
[10] *Bull. No. 5, Hyg. Lab., U.S. Marine Hosp. Serv.*, 1901.
[11] *Ztschr. f. Hyg. u. Infectionskrankh.*, 1905, 52, p. 301.
[12] *Ztschr. f. Fleisch- u. Milchhyg.*, 1908, 18, p. 246.
[13] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1895, 17, p. 742.
[14] Kolle u. Wassermann, *Handbuch d. Path. Mikroorg.*, Ergzheft, 1903, 3, p. 742.
[15] *Arb. a. d. k. Gsndhtsamte*, 1911, 36, p. 198.
[16] *Brit. Med. Jour.*, 1908, 2, p. 1547.
[17] *München. med. Wchnschr.*, 1905, 47, p. 2261.
[18] *Arch. f. Hyg.*, 1906, 55, p. 279.
[19] *München. med. Wchnschr.*, 1907, 64, p. 979.
[20] *Ibid.*, 1909, 56, p. 392.
[21] *Jour. Am. Med. Assn.*, 1909, 52, p. 1455.

Huested[1] on "A Typhoid-like Disease in Rabbits, Produced by the Subcutaneous Inoculation of a Strain of *B. suipestlcus*." The lesions noted by them in this experimental disease are in many respects identical with those obtained by infecting rabbits with the organism isolated by us.

II. SYMPTOMS OF THE DISEASE.

We have, unfortunately, no data as to the percentage of infections or mortality. Of 11 young rats amply exposed to direct contact infection, 8, or 72 per cent, showed at autopsy characteristic lesions of the disease; the remaining 4 rats evidently escaped infection, or were so mildly infected that no traces of the disease could be found. The deaths in the stock cages during the winter months were very numerous, but during the late spring, no rats became spontaneously infected, the epidemic evidently having spent itself. The duration of the disease, as it occurred spontaneously, could not be accurately determined, but appeared to vary within wide limits. In several litters which had been under observation for some time and which were being weighed daily, the duration of the disease, as measured by the initial loss of weight, was 3, 4, 7, and 12 days respectively. These data are only approximate, as the animals were killed when moribund. Moreover, in two rats, characteristic lesions were found altho there had been no previous loss of weight.

In animals experimentally infected and allowed to die, the duration of the illness varied with the mode of infection. Two rats after intraperitoneal injection of a large dose died after two and three days respectively; after subcutaneous inoculation death occurred in six and seven days; after infection by feeding, in nine days.

Emaciation and loss of weight were very marked in most of the rats. There was regularly observed a marked anemia, as judged by the blanching of the ears and the pallor of the eye-grounds. Smears from the blood showed great numbers of normoblasts, and marked polychromatophilia and granular degeneration of the erythrocytes. The normal pink color of the eyes was often changed to a brownish or chocolate tinge by which the disease could be readily diagnosticated. This has been shown by Boycott[2] to be due to methemoglobinemia. A bloody crust about the nose and eyes was always present in the terminal stages. Diarrhea occurred in

[1] *Jour. Med. Research.* 1913, 28, p. 41. [2] *Jour. Hyg.*, 1911, 11, p. 443.

some, but by no means in the majority of the cases. Many of the rats showed no intestinal disturbances, and voided normal dry globular scybalae until their death.

III. BACTERIOLOGICAL FINDINGS.

A bacteriological study was made of three rats dying of spontaneous infection during this epidemic. The cultures obtained were compared with one another morphologically and biologically to establish their identity, and were then inoculated into four rats and fed to two others to fulfill Koch's postulates for determining the etiological relationship of a microorganism to a disease. Other animals were also experimentally inoculated. The strains isolated were also compared with the following cultures:

1. *B. enteritidis* Gaertner, obtained from the collection of the American Museum of Natural History, New York City.

2. *B. typhi murium* A, also obtained from the American Museum of Natural History, is a subculture from a strain isolated by S. Miggi from the "Liverpool virus," a commercial rat poison sold in England and in this country.

3. *B. typhi murium* B, a strain from our laboratory collection.

4. Bacillus from the Rockefeller Institute, isolated during a rat epidemic by Dr. F. Haines.

5. *B. paratyphosus* B. (Schottmüller), from the laboratory collection.

6. *B. coli communis*, from the laboratory collection.

7. *B. typhosus* (James), from the laboratory collection.

8. *B. typhosus*, isolated by one of us from a patient with typhoid fever.

METHODS EMPLOYED IN THE COMPARATIVE TESTS.

Indol and nitrate cultures were examined four, six, and fifteen days after inoculation and were then kept for a few weeks. The observations on the sugar fermentation tubes were made after 48 hours. All fermentation comparisons were made with tested sugars in sugar-free broth. Four degrees of gas production were noted, namely + = trace of gas; ++ = one-fourth of closed arm full of gas; +++ = half-closed arm full of gas; ++++ = three-fourths of closed arm full of gas. Negative control tubes were used in all tests.

Agglutination tests were invariably made by the macroscopic method. Emulsions of the bacteria of approximately the same degree of opalescence were made in normal saline solution from agar cultures grown at 37° for 18 hours. The agglutination readings were taken after incubating the tubes for two hours, and again after four hours; they were then kept at room temperature, and a third reading was taken the next morning. The four-hour reading is used in the following table. In all cases the highest dilution of the serum at which agglutination was visible to the naked eye was taken as the agglutination limit of the serum. Control tubes containing only bacterial emulsion and normal saline were used in every experiment. The serum used was

monovalent and was obtained from a rabbit which had received five injections of dead, and five of live, bacteria. The injections were made daily, with four days' interval between administering the dead and the live bacteria. The bacteria used for immunization were of the strain isolated from Rat A2. Three degrees of agglutination were noted, namely, + = complete reaction; ± = partial reaction; − = negative reaction. All tests were made in duplicate.

The Investigation of the Rat Epidemic.

Rat A1 had profuse diarrhea, and there were crusts of blood about eyes and nose. At autopsy, there were obtained from the heart blood, liver, and spleen pure cultures of a gram-negative bacillus. This was slender and actively motile, resembling morphologically the typhoid bacillus.

Rat A2 was examined about six hours after death. From the heart blood, liver, and spleen in all plates, pure cultures were obtained of an organism identical morphologically and culturally with the gram-negative bacillus found in Rat A1.

Similarly Rat A3 was examined and pure cultures of this organism were again obtained from the heart blood, liver, and spleen. Other rats were examined during the epidemic and gram-negative bacilli were found in all, but a complete cultural identification was not made.

The bacilli were readily isolated in pure culture from all the rats examined.

Morphological and Cultural Characters.

The bacillus is slender, actively motile, gram-negative, and non-spore-bearing. It stains with the ordinary laboratory dyes. In the tissues of the rats the bacillus appeared much larger than in smears from the cultures and showed bipolar staining. On agar plates, the organism grew rapidly, forming moist grey-white colonies which were large and round with smooth borders. On agar slants the growth had the same grey-white moist appearance. The water of condensation was cloudy with a heavy sediment for the first few days; after standing for a week, this cleared up for the most part.

Gelatine stab-cultures showed a fungiform growth and were not liquefied in 30 days.

Broth was evenly clouded in 24 hours. Afterward a heavy

sediment and a thin pellicle formed. At the end of four days the sediment collected in large flakes at the bottom of the tube.

Nitrates were reduced to nitrites. In Dunham's peptone broth, there was slight indol production after four to six days in nearly all tests.

On potato, a scant grey-white growth was obtained that became visible only after three to four days.

Litmus milk at the end of 24 hours was acidified, at the end of 48 hours neutral, and at the end of 3 days alkalin. It was not coagulated.

B. enteritidis Gaertner and *B. typhi murium* A were compared with the organisms isolated from the rats on all the culture media just described. No marked difference was found in any of their cultural characters.

The bacilli isolated from the rats were also compared on serum water carbohydrate media and on fermentation tubes, with *B. typhi murium* A, *B. typhi murium* B, and the rat bacillus from the Rockefeller Institute. Our first series of comparisons were made in April, 1913, very soon after isolating the organisms from the rats. Fermentation tubes were then inoculated with the first subcultures of A1 and A3. A2 had been transplanted about 15 times. The organisms taken for comparison were from stock cultures. The results are as follows:

TABLE 1.

SUGAR FERMENTATION REACTIONS.

Strains	Rat B (A1)	Rat B (A2)	Rat B (A3)	*B. typhi murium* A	*B. typhi murium* B	Rat Bacillus from Rockefeller Institute
Dextrose	Acid	±	Acid	+	+++	++
Maltose	+*	+	+	+	++	++
Levulose	+	+	+	++	++	++
Lactose	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin
Mannite	+	+	±	+	++	+++
Saccharose	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin
Dextrin	"	"	"	"	"	"
Galactose	+	+	+	+	++	++

* Sign + indicates gas production in fermentation tubes; the number of + signs indicates the degree of gas production.

These results were published in our preliminary paper on this subject. Since that time, about 30 daily transplantations on

glucose agar slants have been made, and we have again compared the gas production with the results tabulated:

TABLE 2.

SUGAR FERMENTATION REACTIONS.

Strains	Rat B (A_1)	Rat B (A_2)	Rat B (A_3)	*B. typhi murium* A	*B. enteritidis* Gaertner	*B. typhi murium* B	Rat Bacillus from Rockefeller Institute
Dextrose	+++	+++	+++	+++	+++	+++	+++
Maltose	+	+	+	+	+++	+++	+++
Levulose	++	++	++	++	++	++++	++++
Lactose	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin
Mannite	+++	++++	+++	++++	++++	++++	++++
Saccharose	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin	Alkalin
Dextrin	"	"	"	"	"	"	"
Galactose	++	+++	++	++	++	++++	++++

As may be seen by comparing the two tables, all cultures showed a greater degree of gas production after growing on glucose agar for about two months. All three strains of the rat bacillus produced abundant gas in the glucose fermentation tubes. The rat bacillus from the Rockefeller Institute and *B. typhi murium* B differed from the others only in the degree of their gas production. The three strains of our rat bacillus, *B. typhi murium* A, and *B. enteritidis* Gaertner agreed in every instance except in the degree of gas production in maltose.

AGGLUTINATION REACTIONS.

The agglutinability of our three strains of organisms was compared with strains of *B. typhosus* (James), *B. typhosus* (v. W), *B. paratyphosus* B (Schottmüller), *B. coli communis*, *B. enteritidis* Gaertner, *B. typhi murium* A, *B. typhi murium* B, and the rat bacillus from the Rockefeller Institute. The results are tabulated in Table 3.

These agglutination reactions indicate that the organisms isolated from the rats differ markedly from *B. coli*, *B. paratyphus* B, *B. typhi murium* B, and the Rockefeller Institute rat bacillus. It may also be seen that the two strains of *B. typhosus* agglutinated in quite high dilutions. This curious phenomenon has also been reported by Hübener,[1] Lebram,[2] and others. They have found that

[1] *Fleischvergiftungen u. Paratyphus Infektionen*, Berlin, 1910.

[2] *Ztschr. f. Hyg. u. Infectionskrankh.*, 1909, 44, p. 411.

TABLE 3.

AGGLUTINATION REACTIONS.

Agglutination with Serum of Rabbit Immunized with Rat Bacillus No. A2. Time of Recorded Readings: 4 Hours.

Strains	Rat B (A1)	Rat B (A2)	Rat B (A3)	*B. typhi murium* A	*B. enteritidis* Gaertner	*B. typh.* (James)	*B. typh.* (V. W)	*B. typhi murium.* B	Rat B. Rockefeller Institute	*B. coli*	*B. paratyph.* B
Dilution											
1– 20	+	+	+	+	+	+	+	–	–	–	–
1– 80	+	+	+	+	+	+	+	–	–	–	–
1– 120	+	+	+	+	+	+	+	–	–	–	–
1– 200	+	+	+	+	+	+	+	–	–	–	–
1– 250	+	+	+	+	+	+	+	–	–	–	–
1– 300	+	+	+	+	+	+	+	–	–	–	–
1– 600	+	+	+	+	+	–	+	–	–	–	–
1– 1,200	+	+	+	+	+	–	–	–	–	–	–
1– 2,500	+	+	+	+	+	–	–	–	–	–	–
1– 5,000	+	+	+	+	+	–	–	–	–	–	–
1–10,000	+	+	+	+	+	–	–	–	–	–	–
1–15,000	+	+	±	+	±	–	–	–	–	–	–
Saline Control	–	–	–	–	–	–	–	–	–	–	–

the typhoid bacillus at times agglutinates almost up to the titer limit of the serum of *B. enteritidis* Gaertner.

These agglutination experiments seem to confirm the results of our culture comparisons and suggest that the organism isolated from the rats, *B. enteritidis* Gaertner, and the organism of the Liverpool virus are very closely allied.

The close relationship of the organism of the Liverpool virus and *B. enteritidis* Gaertner has previously been reported by Steffenhagen[1] who made an extensive investigation to determine this fact. He reported that they agreed in practically all respects. The work of Mühlens, Dahm and Fürst,[2] von Ermengen,[3] Schern,[4] and others suggests that both in their cultural characteristics and in their agglutination reactions the organisms isolated by them from rat epidemics agreed with the Danysz, Ratin, Issatschenko, and Gaertner bacillus.

Bainbridge[5] also reports that *B. enteritidis* Gaertner and the Danysz bacillus, which can be easily distinguished from *B. paratyphus* A, *B. paratyphus* B, *B. Aertryck*, and *B. suipesticus* by their agglutination reactions, are indistinguishable from one another

[1] *Op. cit.*

[2] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1909, 48, p. 1.

[3] Kolle u. Wassermann, *Handbuch d. Path. Mikroorg.*, 1903, 2, p. 637.

[4] *Op. cit.*

[5] *Op. cit.*

and apparently, also, are merely strains of the same organism. He also reports that *B. typhi murium* has no existence as a definite organism, since different strains alleged to be *B. typhi murium* and obtained from accredited sources were found to differ greatly. His reports seem to agree with our findings.

It is therefore evident that the bacillus which we have isolated from this epidemic agrees in nearly all respects with *B. enteritidis* Gaertner and with *B. typhi murium* A which we secured from the American Museum of Natural History and which is a subculture of a strain originally isolated from the Liverpool virus, a commercial rat poison sold in England and this country.

This Liverpool virus has also been investigated by Handson, Williams, and Klein.[1] An epidemic broke out in a large business house in London. Twelve persons were taken ill with the disease. Ten days later when they were all convalescent, an investigation revealed the fact that, altho these men all ate at the business house with many other employees, only those who ate in a certain room were taken ill. In this room a bad odor was noticed, and upon removal of the floor boards, 40 dead rats were found. It was then discovered that a rat poison, the Liverpool virus, had been spread on bread and placed around the room so that the rats could get it. Cultures were made from the dead rats and from the patients, and Klein reports that the bacilli isolated agreed in every respect with each other, and with the organism found in the Liverpool virus. Blood sera of the convalescent patients agglutinated the organisms of the Liverpool virus, the organism isolated from the dead rats, and the organism isolated from the patients.

During an investigation of mouse typhoid, Mayer,[2] who was conducting the work, was taken violently ill. Upon examination of his stools mouse typhoid bacilli were isolated. His blood serum in high dilutions agglutinated the mouse typhoid bacilli. He therefore concludes that mouse typhoid bacilli can cause acute and violent illness in man.

Four separate outbreaks of gastro-enteritis, with one death, have been observed by Shibayama[3] in Japan, who shows reasonable grounds for attributing them to the careless use of a rat virus containing Löffler's *B. typhi murium*.

[1] *Op. cit.* [2] *Op. cit.* [3] *Op. cit.*

ANIMAL INOCULATIONS.

In order to establish the etiological relationship of the organism to the disease, we inoculated six rats brought into the laboratory especially for these tests from a lot that had no record of disease among them and which were kept under observation for several days.

Two rats were inoculated intraperitoneally with 0.5 c.c. of a saline suspension of the bacilli grown on agar for 24 hours. These rats died in two and three days. They developed no characteristic symptoms.

Two others were inoculated subcutaneously with the same amount of bacterial suspension; they had no diarrhea, but bloody crusts about eyes and nose formed in five days and the animals were dead in six and seven days.

The last two were fed with bread soaked in 24-hour broth culture; they developed all the characteristic symptoms and died in nine days.

At autopsy, the organisms were recovered in pure culture in all six experimental tests from the heart blood, liver, and spleen, and the characteristic lesions were present.

These results therefore clearly establish the etiological relationship of the bacilli to the disease.

We then inoculated a rabbit, a guinea-pig, and two mice, one intraperitoneally and one subcutaneously with a saline suspension of the bacilli. One c.c. of the bacterial suspension killed a rabbit in three days, 1 c.c. killed the guinea-pig in 18 hours, and one-third of a cubic centimeter killed the two mice in 18 hours.

In every case, the organisms were recovered in pure culture and in smears from the heart blood of the animals; large numbers of the bacilli were found, showing that the organism had developed, giving rise to a true infection. It was difficult to find the bacilli in smears from the heart blood of the rats dying of spontaneous infections, altho they grew in cultures. We also inoculated a rabbit with the same amount of bacterial suspension which had been given to the other animals, killed by heat, as a control for the virulent inoculated material of the cultures. The rabbit was not killed.

As yet we have not tried to infect other animals by feeding.

The majority of workers, however, have not been able to reproduce the disease in rabbits, cats, and dogs by feeding them with the bacillus isolated from epidemics in rats.

Conclusions.

The organisms isolated from the rats proved to be etiologically concerned in causing the epidemic. They were found to agree with the bacillus of the Liverpool virus and *B. enteritidis* Gaertner, both in their cultural characters and in their agglutination reactions. This suggests the probability that all these bacilli are very closely related to one another, if not strains of the same organism. It would seem, further, that the use of commercial rat viruses containing these organisms may be a menace to man.

IV. PATHOLOGICAL FINDINGS.

1. Previous Observations.

The lesions observed by Löffler[1] in the mouse epidemic described by him were enlargement of the spleen, necrotic foci in the liver, and hemorrhagic gastro-enteritis. The Peyer's plaques and the mesenteric lymph-nodes were swollen and congested. Masses of bacilli were always found in the necrotic areas in the liver, but occurred also in the liver capillaries apart from the necroses.

Schilling[2] in 1902 published an account of a spontaneous epidemic among laboratory rats. The duration of the illness was only one or two days, the rats showing weakness, roughness of the hair, closing of the eyes by crusts, and in most cases diarrhea. Retardation of growth was observed in those rats which escaped the disease. Pathologically, there was found enlargement of the spleen. The stomach was normal, but the middle portion of the small intestine exhibited various grades of inflammation and edema, with swelling of the follicles. In the mild cases, the intestinal contents consisted of thin fluid, with gas bubbles; in the severe cases, of mucus admixed with blood. The lower portion of the small intestine, the cecum, and colon were unchanged. The lungs in the acute cases, showed merely petechial hemorrhages and small areas of collapse, but in the more protracted forms of the disease, the lungs were dense, infiltrated, and contained yellowish areas of necrosis. Schilling thus distinguishes two forms of the disease, one acute, with predominantly intestinal lesions, the other more chronic, with marked alterations of the lungs.

Trommsdorf[3] in 1903 gave a fairly detailed description of the lesions produced in white rats by feeding bacilli isolated from the feces of patients suffering from an epidemic of dysentery. These bacilli were agglutinated by mouse-typhoid serum, and were considered by Trommsdorf to be identical with *B. typhi murium*.

The spleen in the infected mice was enormously enlarged, and usually firm. The liver was dark red and showed fatty areas, or was sprinkled with yellowish points. The kidneys were normal. The small intestine was edematous, injected, and filled

[1] *Op. cit.* [2] *Op. cit.* [3] *Op. cit.*

with fluid feces. The mucosa was sprinkled with hemorrhages. The mesenteric, inguinal, and axillary lymph-nodes were swollen and hemorrhagic. The lungs were normal. Bacilli were found in smears from liver and spleen and in cultures from all organs.

Bahr[1] in 1905 made a comparative study of the Danysz and Issatschenko viruses, of a commercial virus called "Ratin," and of *B. typhi murium*. Incidentally, he studied the lesions produced by feeding brown rats with cultures of "Ratin." The lesions noted are the same as those recorded by previous observers, save that Bahr mentions the occurrence of fibrinous peritonitis.

Trautmann[2] in 1906 described the lesions found in epidemic and sporadic infections among laboratory rats at the Hamburg Hospital, and emphasized the resemblance of the clinical picture to that of rat plague. The autopsy findings in both spontaneously and experimentally infected rats were emaciation, hemorrhagic bubos, swelling and punctiform necroses of the liver, marked enlargement of the spleen, hyperemia of the lungs, often with necrotic foci. In feeding experiments, there was swelling of the Peyer's plaques and of the mesenteric, retroperitoneal, and submaxillary lymph-nodes. No microscopic studies were made.

In 1906, there appeared also an exhaustive paper by Kutscher and Meinicke,[3] dealing with the comparative cultural and biological characters of numerous strains of *B. paratyphus* A and B, *B. enteritidis* Gaertner, and *B. typhi murium*. The pathogenicity of the various strains was tested on mice, rabbits, and guinea-pigs, but aside from the gross lesions noted by previous observers, no detailed pathological descriptions are given.

Schern[4] in 1909 studied an epidemic among tame laboratory rats in which the obvious signs of illness lasted only two to three days. The disease was characterized by emaciation, loss of appetite, and in some animals by profuse diarrhea. The lesions found were injection of the peritoneum, without inflammatory exudate, marked enlargement of the spleen, cloudy swelling of the liver, with punctiform red areas beneath the capsule and on section. In the spleen and liver of animals which survived the infection for a longer period, there were pinhead-sized greyish or greyish-yellow foci, suggesting tubercles. The stomach was normal. The intestine was distended, the mucosa in the greater number of cases swollen and congested. The Peyer's plaques were conspicuous. Kidneys were cloudy and swollen, the adrenals slightly reddened. In two rats, large nodules suggesting "pseudo-tubercles" were present in the lungs.

Steffenhagen[5] in 1911 made a careful study of the gross lesions in rats fed with the Liverpool virus, which he considers identical with *B. enteritidis*. Like all the observers quoted, he records marked enlargement of the spleen, which is described as greyish black or reddish grey, with a firm "amyloid-like" consistence; rarely, there was found a soft, hyperplastic splenic tumor. The intestines were distended, the venules of the serosa and mesentery injected. The small intestine contained pale-yellow or thin dark-brown fluid, in which red blood cells could be found microscopically. The mucosa was unchanged but the follicles were swollen. The large intestine was not altered. The lungs were dirty grey on section, very bloody, and succulent. There were pinhead-sized hemorrhages beneath the pleura, and often greyish nodules suggesting tubercles. These consisted of necrotic areas with round-celled infiltration.

[1] *Op. cit.*
[2] *Op. cit.*
[3] *Op. cit.*
[4] *Op. cit.*
[5] *Op. cit.*

Here and there were found pepper-grain sized abscesses with minute metastatic abscesses in the surrounding area. Bacilli were not found in smears from these, so that the origin of the pulmonary lesions was questionable. The liver was enlarged, brownish red, bloody on section, showing irregular, yellowish patches; in advanced cases, the entire liver was of a bright yellow color save for small reddish patches. The cervical lymph-nodes were found enlarged in one case. The adrenals were either diffusely hyperemic or showed a hyperemic zone about the medullary portion.

Boycott[1] in 1911 made the interesting observation that infection of white rats with the Gaertner bacillus was frequently accompanied by methemoglobinemia. The other lesions noted by him, were necroses of the liver and spleen, and, in five rats, acute suppurative myocarditis of the left ventricle.

The foregoing citations include, we believe, the more important contributions to the pathology of the disease caused in rats and mice by bacilli of the paratyphoid and enteritidis group. To this list should be added the study of the liver lesions in rabbits and white mice following infections with *B. suipestifer*, by Boxmeyer,[2] and the recent work of Ordway, Kellert, and Huested, also with a strain of the hog-cholera group. These we shall consider in discussing the genesis of the liver lesions.

2. Gross Lesions.

The gross lesions noted in the present epidemic agree quite closely with those recorded by the writers cited above. No differences were observed between the spontaneously and the artificially infected rats, nor between those infected by subcutaneous injections and those in which the disease was reproduced by feeding. The intestinal lesions, in particular, were not more striking in animals infected by the intestinal route.

Emaciation was always more or less marked. The peritoneal cavity was always free from exudate, even in rats injected intraperitoneally. Marked hyperemia, as described by Bahr and Schern, was not seen. The liver was pale and friable, and sometimes mottled. In some cases, minute, translucent, greyish foci could be detected with the magnifying glass, but were not visible to the unaided eye. The spleen was greatly enlarged in all cases but one, in which it measured only 27 mm. as against 33 mm. and 32 mm. in the healthy controls of the same litter. This exceptional finding will be discussed later. A typical example is Litter D, in which the infected spleens measured 37, 40, and 39 mm.; that of a healthy control rat of the same litter, killed at the same time, 23 mm. The spleens were firm and tense, very dark bluish purple, but irregularly mottled on section by hemorrhagic and greyish areas.

[1] *Op. cit.*

[2] *Jour. Med. Research*, 1903, N.S., 4, p. 146.

The stomach showed nothing abnormal. The intestines were usually collapsed. The intestinal contents in those animals which had previously had diarrhea were fluid, sometimes admixed with mucus, and occasionally flecked with bright blood. Other animals showed normal intestinal contents, with formed scybalae in the large gut. A few scattered petechial hemorrhages were occasionally seen, but no lesions which could be justly described as a hemorrhagic enteritis. The appearance of the Peyer's plaques varied. In the majority of the cases, they were pale and not abnormally prominent. In one case, only, was there found a small ulcer, and this proved on microscopical examination to be a localized destruction of mucous membrane by a rather extensive submucous hemorrhage.

The mesenteric lymph-nodes were not noticeably enlarged, and were not hyperemic. The lungs, with the exception of occasional small purpuric spots beneath the pleura, showed no gross change. One rat, which had been infected by feeding, showed marked alterations in one lung, the relation of which to the inciting organism was not proven. The lung in this case was shrunken, firm, and riddled with caseous abscesses of large size. Microscopically, there was an interstitial pneumonia, and bronchiectatic cavities filled with necrotic exudate. Whether this isolated finding is related to the disease, we are unable to say, as no bacteriological examination of the lung was made, altho the organism was readily recoverable from the blood and spleen in this case. The lesion is apparently the same as that described by Schilling,[1] and according to Currie,[2] is a not infrequent finding in rats apart from the disease which we have studied. The fact that a similar bronchiectatic cavity, lined with squamous epithelium, was found in a rat killed three days after infection would make it improbable that the lesion is associated with the disease.

The heart muscle was sometimes pale, and in one spontaneously infected rat showed macroscopically visible opaque, greyish areas in the left ventricle. The blood was often strikingly thin, and sometimes of a distinct brownish tinge.

Swelling of the cervical and mediastinal lymph-nodes was quite

[1] *Op. cit.*

[2] *Bull. No. 30, U.S. Pub. Health and Mar. Hosp. Serv.*, 1910, p. 55.

regularly found. No gross changes were observed in the kidneys, pancreas, testes, adrenals, or brain. The thymus was usually atrophied and the interlobular septa edematous.

3. Histological Lesions.

Liver.—In both spontaneously and experimentally diseased rats the liver was the seat of numerous focal necroses. These were present in all the cases examined, with the exception of three rats killed on the day following subcutaneous inoculation. The smallest and earliest lesions (30 hours after injection) appeared as agglomerations of distorted nuclei, lying in a fibrinous meshwork in which were entangled a few red blood cells. In the older lesions, the nuclear constituents gradually disappear; there remains a sharply outlined, roughly circular area, almost devoid of cells, staining intensely with eosin, and showing a swollen nodular framework of fibrin. In places the interlacing nodular strands of fibrin appear to fuse into hyaline masses. The fibrinous material stains orange red with Mallory's connective tissue stain, and bluish with Weigert's fibrin stain.

The necrotic areas are at once made evident, in sections stained with Sudan III, by the abundant accumulation of fat drops. These are of largest size and most numerous at the margin of the necroses. The central portion may contain a few finely divided droplets, or may be virtually free from fat. Here and there are small groups of liver cells laden with fat. These are probably at the edge of necrotic areas which did not appear in the sections. Fine fat droplets are also found free in the capillaries and within the Kupffer cells.

Apart from the necrotic areas, the liver cells show no degenerative changes. In some of the rats, notably in one killed 24 hours after subcutaneous inoculation, mitoses are very numerous, but not especially so about the necroses.

Within the liver capillaries, are found in great number abnormal cellular elements and occasional fibrin thrombi. The cellular structures are, for the most part, large mononuclear cells with basophilic cytoplasm, and relatively large, kidney-shaped nuclei. The fewest of these are normal and the greater number more or less

degenerated. They very commonly inclose pyknotic nuclear particles; sometimes the fairly preserved remnant of a lymphocyte, sometimes one or more red cells, rarely a group of bacilli. One finds also unphagocyted chromatin fragments and small cells with ring- or signet-shaped nuclei, obviously lymphocytes in various stages of

FIG. 1.—Liver of *Rat C1*, spontaneously infected, showing necrotic areas (*a*), and large masses of cells in a sub-lobular vein (*b*).

pyknosis. In some of the sections, cellular elements and detritus of this sort are extremely abundant, and appear to block the capillaries, especially when they become inclosed in a fibrinous matrix. We shall discuss the relation of these cell fragment thrombi to the origin of the necroses farther on.

The behavior of the Kupffer cells was somewhat variable. In some of the rats they were swollen and vacuolated, sometimes exfoliated, and frequently contained inclusions of nuclear material or erythrocytes. In other rats showing equally extensive areas of necrosis, the Kupffer cells showed little or no change. In one infected rat which was vitally stained with Trypanblau very few

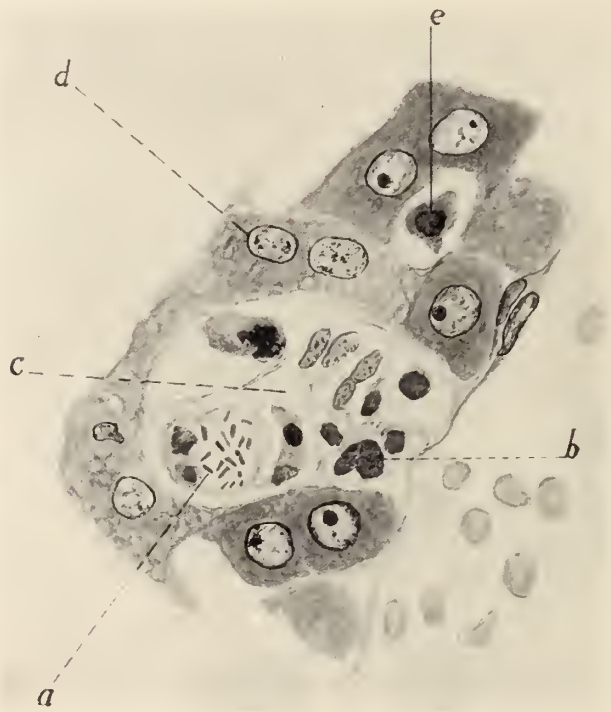

FIG. 2.—Early cell fragment thrombus in liver. At (*a*) mononuclear phagocyte enclosing bacilli; *b*) degenerating mononuclear cells; (*c*) fibrin net.

of the cells lying free in the capillary lumina contained blue granulae. There was no noticeable accumulation of the stained cells about the necroses or within them. Those endothelial cells which lay within the necrotic areas were obviously degenerated. The blue-staining material was in the form of large clumps instead of granulae of fairly uniform size. So far as one can judge from this single experiment, it would seem that the Kupffer cells are apparently not

the chief source of the foreign cellular elements and cell detritus in the capillaries.

All the different forms of cell fragments, phagocytes, and intact mononuclear cells are found in the portal vein, and at times in the efferent veins of the lobules. They occur singly or in large

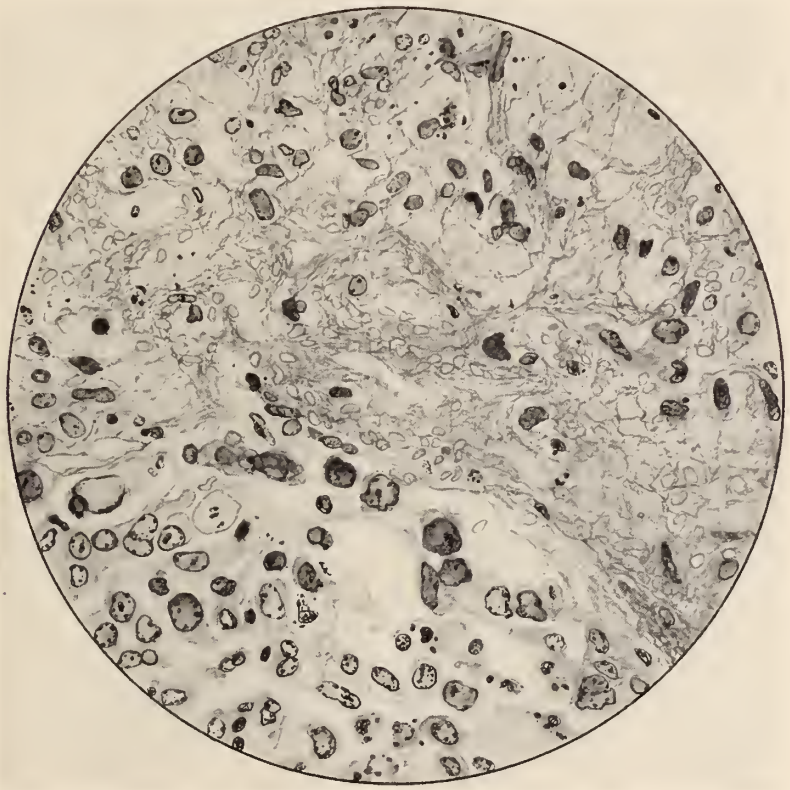

FIG. 3.—Spleen of *Rat B-2-x*, experimentally infected. Section includes edge of follicle. Note fragmentation of nuclei, especially in pulp, and abundant exudation of fibrin.

clumps, in which the cellular constituents are imbedded in a fibrinous matrix.

Spleen.—In this organ, the lesions are localized in the early stages, more diffuse in animals which survive the infection for a longer period. The essential, and probably the primary change appears to be an intense destruction of the lymphoid cells both with-

in and without the follicles. Throughout the pulp, one finds small mononuclear cells in all stages of disintegration, as evidenced by the abundance of fragmented chromatin. Much of this nuclear detritus is within macrophages, which in turn undergo degeneration. The necrosis of the pulp cells is accompanied by a great outpouring of fibrin, forming a nodular network about the degenerating cells. The filaments of fibrin appear to use the fibrous reticulum as a support. There is often distinct hemorrhage, but rarely excessive pigment. The follicles show invariably a more or less marked pyknosis of the small lymphoid cells. This is accompanied in the earlier stages by an excessive formation of large mononuclear cells. The number of mitoses among these may be extraordinary. Phagocytosis of chromatin fragments by the larger cells of the follicles is of course regularly seen. No fibrin could be demonstrated within the follicles.

Bacilli are found in small groups or in larger clumps both within and without the sinuses. In spontaneously infected rats, or in those infected by feeding, very few bacteria may be present. The sinuses are crowded with red cells, but they may contain impacted masses of mononuclear cells or cell-débris, about which there may be formed a definite fibrin thrombus. Masses of cells are also commonly found in the splenic veins. Among these are cells which may undergo mitotic division in the blood stream. Such cells were also found several times in smears made directly from the splenic blood, in which one finds also numbers of nuclear fragments and phagocytes enclosing red cells and chromatin particles.

Kidneys.—In animals experimentally infected with subcutaneous or intraperitoneal injections, it is usual to find hyaline thrombi in the glomerular capillaries. Every tuft may show a more or less complete blocking. The appearance is especially striking in sections stained with Mallory's anilin blue, the hyaline material taking a reddish-orange color in contrast to the yellow of the red blood cells. Similar thrombi may be found in the capillaries of the medulla and pyramids.

Bacterial emboli in the glomerular loops and in the capillaries between the tubules were seen in two rats inoculated intraperitoneally, but were not regularly present.

Myocardium.—Lesions in the myocardium were found both in the spontaneously and experimentally infected rats, tho not in all cases. The changes were in the nature of an acute interstitial inflammation, and occurred by preference in the superficial strata

FIG. 4.—Myocardium of left ventricle, showing acute interstitial inflammation in spontaneously infected rat.

of fibers, more especially in the left ventricle. Rat A (spontaneously infected) showed extremely marked lesions (Fig. 4). The fibers were forced apart by a cellular exudate and fibrin. Most of the inflammatory cells are fragmented beyond recognition, but at the margin of the lesion, it can be seen that there is swelling and degeneration of the fixed connective tissue cells, as well as a moderate infiltration of lymphoid and polymorphonuclear elements.

The muscle fibers show various degrees of degeneration up to complete necrosis. In some areas, the fibers have disappeared completely, leaving only an irregular eosin-staining material, in which are found swollen, distorted nuclei and chromatin fragments. Bacilli occurring singly and in clumps are found without difficulty between the muscle fibers.

Lungs.—The septa appear relatively stout and more cellular than those of normal lungs. This is due to the stuffing of the capillaries with large mononuclear cells similar to those found in the liver. Many of the larger pulmonary arteries contain groups or larger masses of these cells. Pneumonic lesions, aside from these interstitial changes, were not found, and the bronchi were free from exudate. The one exception (Rat D2*x*) has already been described.

Stomach and intestines.—The only lesions found in the stomach were occasional small hemorrhages into the mucosa. The intestinal lesions, on the whole, were not striking, and in some of the rats no definite alterations were found. In others there was moderate fragmentation of the lymphoid cells, with phagocytosis by the swollen reticular elements. The overlying mucosa was usually intact, but not infrequently the villi were edematous, and there were more or less extensive hemorrhages; this had led in one case to a separation and desquamation of the epithelial covering; aside from this purely mechanical loss of substance, no ulcers were found in any of the rats examined.

Lymph-nodes.—Serial sections of the neck organs were made from a number of the spontaneously infected rats for another purpose. In all the cases it was found that the cervical lymph-nodes were greatly enlarged and the seat of extensive necroses similar to those described in the spleen. Altho the thymus exhibited advanced involutional changes such as are found in all infectious diseases accompanied by wasting, in no case did it contain areas of necrosis, even when all the surrounding lymphoid tissue, including that which abundantly envelops the primary bronchi, was extensively affected. The mesenteric lymph-nodes showed slight involvement in some rats; in others, no changes were present.

The marrow of the long bones contained more or less extensive

areas of necrosis (Fig. 5) distributed throughout the shaft. Necroses were also found in the bones of the jaw and clavicle. There seemed in some cases to be an increased number of megakaryocytes present, and in smears from the marrow

FIG. 5.—Section through shaft of femur showing extensive necroses in marrow of *Rat D-4-x*, spontaneously infected.

stained with Giemsa one of these cells containing plural mitoses was seen.

No lesions were found in the thyroid, parathyroid, pancreas, or testes. The adrenals showed no loss of chromaffinity and were normal in other respects. The central nervous system, which showed no gross changes, was not studied microscopically.

PROTOCOLS.

Rat C-1.—This was one of a litter which had been operated upon when 14 days old. The animal gained normally until it became ill, when the weight dropped from 98 gm. to 77.5 gm. The rat became emaciated and refused food, and the eyes were closed with secretion. It was killed by chloroform.

Autopsy: No intestinal lesions were noted. The spleen was enlarged and showed prominent follicles. The liver was large and pale. No other gross changes were observed.

HISTOLOGICAL EXAMINATION.

Liver: Very numerous areas of necrosis, for the most part discrete, but in places confluent. The liver cells at the margin of these finely vacuolated. The necrotic areas are almost devoid of cells, being composed of a coarse fibrinous reticulum, in which are a few lobulated nuclei. In some places, small accumulations of leukocytes, chiefly polymorphonuclear. Dense aggregations of cells in the portal spaces, most of them lymphoid and plasma cells. These often extend in streaks along the interlobular bile-ducts and vessels. The liver capillaries are narrowed apparently by the swelling of the liver cells. They contain degenerated cells which in a few places form definite plugs.

Spleen: With the low power, one sees numerous scattered, rather ill-defined areas, less cellular than the surrounding pulp, composed of a hyaline or granular eosin-staining material deposited in the reticulum about the splenic sinuses. The cells which are included in these regions are pyknotic and fragmented. In places, distinct hyaline thrombi occupy the sinuses in the center of these rarefied areas. About such thrombosed sinuses there are often irregular hemorrhages. The malphigian follicles show slight changes, but phagocytosis of fragmented nuclei is occasionally seen. Mitoses are not numerous. A few megakaryocytes are scattered through the pulp.

Adrenals: Normal. The chromaffin staining is marked.

Kidney: Normal.

Lungs: The alveolar walls thickened showing nodular areas of necrosis. Many of the smaller arteries packed with masses of mononuclear cells.

Rat C-2.—Thymectomy at 14 days. Recovered from operation, and gained steadily in weight until 24 days before death. The weight then remained stationary for 10 days, but fell from 59 gm. to 43.5 gm. during the last two weeks of life. The animal was found dead and partially eaten by the other rats of the litter.

Autopsy: No gross lesions were noted, aside from the extreme emaciation. The spleen measured only 25 mm. in length.

HISTOLOGICAL EXAMINATION.

Liver: Necroses, very abundant and some of them of large size, are composed of a swollen hyaline fibrinous reticulum inclosing a sparse number of fragmented or distorted nuclei and a few red blood cells. The liver capillaries, which are narrow, contain many small chromatin fragments, but no very definite thrombi, nor are larger masses of cells found in the portal veins. The intact liver cells often show large, hyperchromatic nuclei.

Spleen: Very markedly altered, the follicles small and very irregular. The small cells of the follicles are in all stages of pyknotic degeneration. Many large phagocytes filled with nuclear fragments. The pulp shows a striking paucity of cells as compared

with the normal. Between the compressed, and in places, obliterated sinuses, there is a large amount of a granular fibrinous material, imbedded in which are nuclear fragments and red blood cells. The endothelium of the sinuses is well preserved, but the cellular constituents of the pulp are largely disintegrated. Some of the larger sinuses contain hyaline thrombi, others are filled with loose masses of degenerating cells.

Adrenals: Almost complete loss of chromaffin staining, possibly the effect of postmortem change. The cortex is not altered.

Myocardium: In the wall of the left ventricle several small localized areas of hyaline or waxy degeneration of the muscle fibers, and about these, slight cellular infiltration.

Lungs: No noteworthy changes.

Cervical lymph-nodes: Numerous areas of necrosis, located chiefly in the lymph-cords.

In this animal, the disease was apparently of long standing, and had led to extreme destruction of the cellular elements of the spleen, and to very extensive necroses in the liver. There was, however, no attempt at healing or encapsulation, and the lesions may be considered as progressive. An exceptional feature in this rat is the small size of the spleen, due possibly to the advanced destruction of the cellular constituents, or to a secondary contraction of the necrotic areas, such as occurs in an anemic infarct.

The third rat of this litter illustrates the more acute lesions.

Rat C-7.—A control rat, unoperated, which gained weight progressively until killed, showing no abnormal symptoms.

Autopsy: Weight 70.5 gm. The organs appeared normal with the exception of the spleen, which measured 35 mm. in length. The follicles were very conspicuous.

HISTOLOGICAL EXAMINATION.

Liver: A few typical early necroses, some of which are the seat of marked leukocytic infiltration. Definite hyaline plugs are found in some of the necrotic foci. The small sub-lobular veins are surrounded by a mantle of lymphoid and plasma cells. Swelling and some vacuolization of the liver cells. The capillaries are narrow and contain moderate numbers of small cells, but no larger masses of cell fragments, or phagocytes.

Spleen: The pulp intensely congested, and in places, hemorrhagic. A few small areas of necrosis, with fragmentation of the nuclei. The follicles are large, the follicle cells well-preserved. Numerous megakaryocytes are present.

Adrenals: Intense chromaffin staining.

Thymus: Normal.

Rat E-6.—Unoperated control, aged 2 months and 6 days. Duration of disease as estimated from beginning loss of weight was 9 days, during which period the weight fell from 65.5 gm. to 59 gm. The rat was chloroformed, having shown the usual symptoms.

Autopsy: Emaciated. No peritoneal changes. Spleen much enlarged (36 mm.), with conspicuous follicles. Liver shows nothing abnormal. Adrenals, kidneys, lungs, intestines free from gross changes.

HISTOLOGICAL EXAMINATION.

Liver: Sharply circumscribed, very numerous necroses, some of large size, composed of a coarse fibrinous meshwork with occasional red blood cells, and degenerating

cells and nuclear fragments. Such cells are also found free in the capillaries, singly or massed into clumps and enveloped in a delicate fibrinous mesh. The Kupffer cells in places are swollen and contain ingested red blood cells and chromatin particles. Aggregations of large cells in the portal veins and often in the lymph-spaces of Glissons capsule.

Spleen: The section shows numerous scattered areas of necrosis, consisting of a fibrin meshwork inclosing distorted, pale, epithelioid nuclei, erythrocytes, and small particles of fragmented nuclear material. Elsewhere the sinuses are congested and frequently contain masses of large mononuclear cells, few polymorphonuclear leukocytes, and other cell fragments whose origin it is impossible to determine. Definite thrombus formation is not noted. Mitoses numerous, both within the follicles and in the reticulum. A great many megakaryocytes.

Lungs: Throughout the section, irregular, somewhat nodular areas of consolidation, not of the type of a lobular pneumonia, but consisting of cellular infiltrations or proliferations of the alveolar wall, without exudation into the alveoli. In the center of such an area, there may often be found a small pulmonary artery packed with cells which are of the same kind as those seen in the liver capillaries. Large mononuclear cells predominate. They are often vacuolated, and frequently inclose erythrocytes or pyknotic nuclear fragments. The consolidated areas are thus rather definitely distributed about the smaller arterial branches, and not the bronchi which are free from exudate and otherwise quite normal. The capillaries in these areas are not infrequently found plugged by fibrin masses inclosing nuclear fragments.

Myocardium: Most extensive changes (Fig. 4). The fibers are widely separated, there having been evidently an extreme edema of the interstitial tissue. The muscle fibers are thin, and very refractile and waxy in appearance, with complete loss of the transverse striations, and only a suggestion here and there of the longitudinal fibrils. The nuclei are for the most part still fairly well preserved, a few only being pyknotic. Between the fibers are accumulations of wandering cells, the majority of the mononuclear variety. In places, and especially beneath the endocardium, they form dense clusters. There appears to be also a proliferation of the fixed connective tissue cells; altho no mitoses are found, many of the cells between the degenerated fibers are of the fibro-blastic type. These myocardial changes are not uniformly present throughout the heart, but affect particularly the wall of the left ventricle and the papillary muscles.

Organs of the neck: These were cut in series of 10 microns. The thymus was the seat of marked involutional changes. Differentiation between cortex and medulla was lost. The lobules were separated by edematous tissue in which were many mast-cells. The mediastinal and cervical lymph-nodes were enlarged and contained many areas of focal necrosis. The thymus, on the other hand, was entirely free from necroses. The thyroid and parathyroids were normal. The salivary gland was normal.

Bone-marrow (femur): Scattered areas of focal necrosis, distributed throughout the shaft and present also in the lower epiphysis. Megakaryocytes appear to be present in increased numbers.

In all, 10 spontaneously infected rats were examined and a fairly complete microscopic study made. From the last rat examined, and from several other rats infected at about the same time, the bacillus which has been described above was isolated, and a further

study made of the lesions produced by experimental infection with pure cultures of the organism. As has been stated, it has been found to be easy to produce the disease, either by feeding or by subcutaneous or intraperitoneal inoculation. The resulting lesions were in all respects similar to those described in the spontaneously infected rats; the differences found in rats infected by different routes appeared to depend upon the duration of the illness rather than the mode of infection.

The following protocols of experimentally infected rats illustrate the similarity between the naturally and artificially produced disease:

Rat B-1-x.—Inoculated intraperitoneally with 0.5 c.c. of a saline suspension of a 24-hour agar culture. Found dead 48 hours later. No putrefactive changes.

Autopsy: Negative save for slight swelling of spleen. Cultures from peritoneal cavity, heart blood, spleen, and liver, positive.

HISTOLOGICAL EXAMINATION.

Liver: Many small areas of necrosis, scattered through the different portions of the lobules. The smallest, and presumably the earliest of these, appear as irregular plugs of coarse fibrin distending the capillary lumen and inclosing numerous unaltered red blood cells and a few nuclear fragments. In the capillaries are many mononuclear cells, with inclusions of masses of chromatin and red cells (Fig. 2). The Kupffer cells, swollen and vacuolated, also contain ingested nuclear particles and erythrocytes. The origin of most of these phagocytic cells, which are in all stages of disintegration, is impossible to determine. It is obvious that about these masses of cell detritus fibrin thrombi are formed in the sinusoids, and that these cell-fragment thrombi antedate the destruction of the liver cells. Thus one finds occlusion of the capillaries without noticeable alteration of the adjacent liver cells. With the stoppage of a larger capillary area, the liver cells about the thrombosed area become vacuolated, their cell outline indistinct, their nuclei shrunken and irregular in outline; the nucleolus disappears, and the chromatin lies against the nuclear membrane or is extruded into the cytoplasm. With the complete breaking-down of the liver cells, they become incorporated into the thrombotic area.

Many large mononuclear cells, most of them showing karyolytic changes, are present both in the portal and in the efferent veins. Many of the individual cells are phagocytic.

Bacilli are found in clumps in the capillaries, but in no particular relation to the necroses.

Spleen: The follicles irregular, but large and relatively well preserved. The most striking alteration is in the pulp, which even with the low power shows an extraordinary fragmentation of the nuclei (Fig. 3). The meshes of the reticulum are thickly crowded with nuclear particles of all sizes and shapes between which there is hemorrhage and fibrin formation. The picture is complicated, but it is evident that there has been an extreme destruction of the lymphoid cells, both within the follicles and in the reticulum. Coincidently there is taking place a proliferation of large mononuclear cells, with lobate or oval nuclei and basophilic cytoplasm. The follicles con-

tain many healthy-appearing cells of this type, and many of them are in mitosis. They become phagocytic for the pyknotic, small lymphoid cells and after becoming loaded with ingested nuclear material, in turn degenerate. The larger sinuses and veins contain many of these cells and some of them appear to undergo mitosis in the circulating blood. Some of the sinuses are plugged with cell-fragment thrombi, but the majority are filled with well-preserved red blood cells. The endothelium of the sinuses preserves its normal character, shows no mitoses, and contains no phagocytic inclusions. No pictures are found suggesting a differentiation of the sinus endothelium into the large basophilic mononuclear type of cell.

Large colonies of bacilli are scattered irregularly through the splenic pulp. No megakaryocytes are seen in the sections.

Kidneys: Hyaline thrombi present in the glomerular capillaries, forming more or less complete casts of the tufts. They stain intensely with Weigert's fibrin stain. The blocking of the blood-channel is not always complete, the hyaline or fibrinous material being sometimes deposited along one side of the capillary wall. The epithelium of the tuft and of Bowman's capsule unaltered. Occasional hyaline thrombi also found in the capillaries between the collecting tubules. The thrombus may extend for a distance either into efferent or afferent vessel or both.

Adrenals: Normal in all respects.

Intestines: A few hemorrhages into the mucosa. The sections do not pass through the Peyer's plaques. The serosa is free from inflammatory change.

Stomach and duodenum: Normal.

Pancreas: Normal.

Mesenteric lymph-nodes: There is a slight hemorrhage near the surface of the gland, but no necrosis. The changes are insignificant.

Lungs: By oversight, not examined. Section of lung from Rat B-2-*x* inoculated at same time and showing similar lesions in other viscera, may be taken for description. The capillaries are crowded with degenerating cells of the same character as those seen in the liver capillaries. Large mononuclear cells often enclosed in a delicate fibrin network, are also present in numbers in the smaller pulmonary arteries, but are rarely found in the veins. Bronchi and alveoli are normal.

Myocardium: The superficial layer of ventricular muscle is edematous. The fibers are separated by a fibrinous coagulum. There are bacterial emboli in the capillaries.

The following protocol describes the lesions in a rat infected by feeding.

Rat D-1-x.—Fed with cubes of bread soaked in saline suspension of 24-hour agar slant. No food given on following day. The rat showed the usual symptoms and died nine days after infection. On ice, six hours before autopsy.

Autopsy: Marked emaciation. Hyperemia of intestine without marked swelling of Peyer's plaques. Spleen much enlarged, liver moderately. Lungs and heart show no gross changes.

HISTOLOGICAL EXAMINATION.

Liver: Necrotic areas of considerable size present in great numbers, irregularly disposed in various portions of the liver lobules. In these areas, the architecture of the liver is destroyed; one sees merely a very coarse nodular network of fibrin in which lie a few distorted nuclei and nuclear fragments. Some of these appear to have been derived from the pre-existing endothelial cells; others are small and round and resemble

pyknotic lymphocytes. The liver cells immediately abutting on the necrotic areas sometimes show nuclear degeneration, but on the whole, the transition to normal tissue is very abrupt. Apart from the necrotic areas, one finds as in Rat B1*x*, distended capillaries filled with broken-down cellular detritus, enmeshed in fibrin, and about these, changes in the liver cells of varying degree up to complete necrosis. Very striking are the large masses of cellular detritus in the larger efferent veins.

In sections deeply stained with polychrome methylene blue, very few bacilli are found, and these scattered irregularly through the capillaries. They are often inclosed within phagocytes and degenerated. Larger bacterial colonies are not seen.

Spleen: The lesions are less marked than in many other rats examined. The follicles are small and indistinctly outlined. Fragmentation of the lymphoid cells and phagocytosis are not extreme. The changes are more pronounced in the reticulum. The sinuses which are distinct and well filled with red blood cells are widely separated by a fibrinous material, containing distorted nuclei, in different stages of disintegration. Some of the larger sinuses, but especially the larger splenic veins, are filled with masses of cells embedded in a pink-staining matrix.

Kidneys and adrenals: No marked change. No fibrin thrombi in the glomeruli.

Lungs: Lesions are practically identical with those found in Rat B2*x*.

Stomach: Localized areas of intense hyperemia in the superficial layers of mucosa, and red cells are found in numbers in the stomach contents.

Intestines: No changes are found in the mucosa of the small or large intestine. The Peyer's patches are not included in the sections.

Mesenteric lymph-nodes: Quite numerous areas of partial necrosis, in which the lymphoid cells are replaced by large cells with vesicular lobate nuclei, often containing pyknotic nuclear particles in their cytoplasm.

Myocardium: Normal.

In all, 22 rats were inoculated, and a histological study made of the lesions. The above protocols are selected as typical of the early and late stages of experimental infection. They do not, however, include a description of the changes in the Peyer's plaques, a brief record of which may be taken from the protocols of other infected rats.

Rat M-5-1.—Subcutaneous injection. Killed after 47 hours. Very early lesions in spleen and liver.

Stomach: Normal.

Small intestine: The epithelium is intact. Among the lymphoid cells of the Peyer's plaques are very numerous cells of large size, with vesicular, slightly irregular or lobulated nucleus. These cells are in active proliferation: mitotic figures extremely abundant. The small cells well preserved. Only a few pyknotic nuclei.

The only abnormality noted in these sections is the very active proliferation of large mononuclear cells, unaccompanied by marked destruction of the lymphocytes, or phagocytosis.

Rat M-5-2.—Subcutaneous injection. Killed 57 hours after inoculation. Typical marked lesions in liver and spleen. The changes in the small intestine are similar to the above, but mitoses among the large cells are less numerous.

Rat M-12-4.—Splenectomy. Subcutaneous infection. Killed 3 days after inoculation. Typical lesions in the liver.

Small intestine: The changes very slight, and of the same character as in Rats M-5-1 and M-5-2. No necroses or hemorrhages. The mesenteric lymph-nodes show most active proliferation of large mononuclear cells. The sinus endothelium is readily distinguished from these, and tho there is slight swelling and desquamation, no active proliferation is seen.

Rat M-12-5.—Subcutaneous inoculation. Killed after 3 days. Typical lesions in spleen and liver.

Intestines: Peyer's patches large. In a few places, pyknosis of lymphoid cells, with phagocytosis. The large cells show numerous mitoses. The overlying epithelium is unchanged. In some sections, however, there is hemorrhage into the mucosa, with edema as shown by the separation of cells of the stroma and the deposition of granular material. No inflammatory reaction. Sections of several taenia in the villi.

The above brief notes will suffice to show the insignificance of the intestinal lesions. Aside from moderate fragmentation of lymphoid cells, active proliferation of mononuclear elements apparently derived from the reticulum, no changes of note were observed. The presence of parasites in the intestinal villi in Rat M-12-5 may perhaps explain the more marked hemorrhage and edema seen in this case.

LESIONS IN MICE.

The following is a brief abstract of the changes noted.

Mouse 1.—Inoculated intraperitoneally with 0.3 c.c. of a saline suspension of a 24-hour agar slant. Found dead after 18 hours.

Autopsy: Spleen enlarged. Liver pale. Lungs congested. Many bacilli in heart blood and peritoneal exudate.

HISTOLOGICAL EXAMINATION.

Liver: Fibrinous exudate with large masses of bacilli on the surface. Capillaries contain increased numbers of lymphocytes with occasional pyknotic nuclei. No thrombus formation. A few colonies of bacilli in the capillaries.

Spleen: Marked degeneration of the lymphoid cells, both in follicles and in reticulum, with extremely active phagocytosis. There are also extensive hemorrhages into the splenic pulp. Many pigment-containing cells. Many megakaryocytes. No definite exudation of fibrin.

Kidney: Many bacterial emboli in the glomeruli and elsewhere. The glomerular tufts are free from fibrinous thrombi.

Myocardium: Bacterial emboli are present in the capillaries.

Mouse 2.—Inoculated with 0.3 c.c. of saline suspension subcutaneously. Found dying after 18 hours. Killed with chloroform.

Autopsy: Spleen enlarged and congested. No other gross lesions. Many bacilli in smears from heart blood.

HISTOLOGICAL EXAMINATION.

Liver: Same as Mouse 1. No necroses or capillary thrombi.

Spleen: Same as Mouse 1, save that large mononuclear cells in mitosis are very numerous, probably because of fresh preservation. Many megakaryocytes.

Kidneys, lungs, and myocardium are normal.

In both mice, death occurred apparently without the development of marked lesions comparable to those seen in infected rats. A third mouse, inoculated with killed cultures, exhibited marked lesions, which closely resembled those in infected rats.

Mouse 3.—Inoculated intraperitoneally with 0.3 c.c. of culture killed by heating. Found dead after 18 hours.

Autopsy: Spleen markedly enlarged. Liver soft and pale. Other viscera show no changes.

HISTOLOGICAL EXAMINATION.

Liver: The capillaries are packed with chromatin fragments and distorted nuclei of bizarre shape. In many places, these are associated with the formation of definite fibrin thrombi. Necroses of the liver cells are seen in many places, but the appearance differs somewhat from that found in rats in the preservation of the alignment of the necrotic cells. Their nuclei at first become pyknotic, later fragment and disappear; the cells become smaller, denser, more hyaline, and stain intensely with eosin. The necrotic areas are not very circumscribed.

Spleen: Many fairly limited areas of necrosis in the pulp, in which there is extreme nuclear fragmentation. Hemorrhages also.

Lungs: Marked hyperemia. Capillaries stuffed with nuclear fragments.

Mediastinal lymph-node: Fragmentation of lymphocytes and phagocytosis of cell detritus by large cells. Exudation of coagulable material into the lymph sinuses.

Myocardium: Normal.

No feeding experiments were performed with mice, and the intestines were not examined.

LESIONS IN RABBITS.

As was stated in the bacteriological part of this paper, rabbits were found to be susceptible to intraperitoneal and intravenous inoculations with the bacilli. The lesions produced resemble closely the lesions observed in rats, as will be evident from the following protocol.

Rabbit 1.—Inoculated intraperitoneally with 1 c.c. of saline suspension of 24-hour agar slant. Died in 18 hours.

Autopsy: Along lymphatics of anterior abdominal wall, several small cheesy abscesses. No free fluid in peritoneal cavity. The omentum is rolled upon itself and dotted with opaque, whitish exudate. The serosa is not greatly congested. There is fibrino-purulent exudate of a cheesy character on liver, spleen, and intestinal serosa. Liver very large, dark, with distinct lobules having a darker center. The consistence is friable. No macroscopic necroses. No coccidiosis. Spleen moderately enlarged,

dark, firm. Kidneys very soft, friable, and swollen, sprinkled superficially and on section with blotchy hemorrhages. Adrenals: medulla hyperemic. Pancreas normal. Lungs: small pleural and parenchymal hemorrhages. Myocardium, stomach, and intestines normal. Mesenteric lymph-nodes not enlarged or necrotic.

Positive cultures obtained from peritoneal cavity and heart blood.

HISTOLOGICAL EXAMINATION.

Liver: The liver cords are narrow, the capillaries wide, and filled with great numbers of cellular fragments. In many places they are agglomerated into impacted masses imbedded in fibrin, which is readily demonstrated with Weigert's stain. The appearance is identical with that repeatedly described as typical of the livers of infected rats. In addition, however, there are larger areas which have rather the appearance of irregular infarcts. The liver cells still maintain their columnar alignment, but the individual cells are homogeneous and stain intensely with eosin. The nuclei are lost, and the cells evidently completely necrotic. The blood channels in these areas are not occluded with thrombi, but in some places are plugged with masses of bacilli. The entire necrotic portion which involves several lobules, is walled off by a zone of leukocytic infiltration. In the absence of serial sections, it was not determined whether there was a blocking of larger vascular channels.

Spleen: The striking feature is the presence of dense thrombi composed of fibrin, red blood corpuscles, and nuclear fragments in many of the splenic sinuses. There are areas of hemorrhage in the pulp. The follicles show changes which are the counterpart of those noted in rats.

Lungs: There is an unorganized fibrin thrombus in one of the larger branches of the pulmonary artery. This does not show the structure of a typical platelet thrombus, but appears to be composed of irregular laminae of fibrin, and contains dense aggregations of chromatin dust, in its central portion. Similar thrombi are present in smaller arterial branches near the surface and are associated with hemorrhage into the alveoli. The capillaries are crowded with leukocytes, among them many polynuclears. There is no exudate into alveoli or bronchi.

Stomach: Normal.

Small intestine: Sections include a Peyer's plaque. The mucosa is unchanged. Rarefaction of the centers of the follicles and fragmentation of the nuclei of the lymphocytes leading to their complete disappearance in some regions. Their place is taken by large mononuclear cells, also in various stages of degeneration, and containing yellowish pigment.

Myocardium, adrenals, and kidneys show no changes.

V. GENERAL CONCLUSIONS.

The disease, as exemplified by the foregoing description, presents features which are of considerable interest to the pathologist. The origin of the necroses in spleen, liver, lymph-nodes, and bone-marrow, which constitute the most striking alterations, demands explanation. Mallory and Ordway have already emphasized the resemblance of this rat disease to human typhoid in the character

of the lesions, and its suitability for the experimental study of some of the disputed points in the pathology of typhoid fever.

Two divergent opinions as to the origin of the liver necroses in human typhoid have been expressed. It has been held, on the one hand, that the lesions are the direct result of the action of bacterial toxins or of the bacteria themselves upon the parenchymal cells. Thus Reed[1] concludes, from a study of human lesions and necroses produced by the injection of typhoid bacilli into the mesenteric veins of rabbits, that the areas of necrosis "owe their origin to the action of the typhoid bacillus, altho it has not been possible to determine definitely in what way this cell death is brought about, that is to say, whether it is due to the immediate presence of the bacilli within the areas of necrosis, or is caused by the action of the so-called toxalbumins which are assumed to be present in the general circulation." He considers the latter more probable because of the experiments of Welch and Flexner[2] with diphtheria toxin and of Flexner[3] with minute injections of ricin.

The alternative view is that of Mallory,[4] who holds that the liver necroses in typhoid fever are the direct result of the embolic occlusion of liver capillaries by impacted masses of cells and cell-fragments, largely of endothelial origin ("endothelial leukocytes"), which are swept into the portal circulation from the spleen, intestine, and mesenteric lymph-nodes. The typhoid toxin acts primarily as a direct stimulant to the proliferation of the large mononuclear cells in these tissues. These new-formed cells are short-lived, and in their disintegration give rise to the cell fragment emboli in the liver and other organs.

In the lesions described, we have repeatedly noted the presence of great numbers of cell fragments in the liver capillaries, and of larger masses of cells in the portal vessels. Altho the appearances seemed to point to a capillary occlusion as the primary event, and the necrosis of the liver cells as a later effect, it seemed of interest to establish this point experimentally, if possible.

The first question to be determined was whether, in the earliest lesions, there could be shown a complete blockage of the capillaries

[1] *Johns Hopkins Hosp. Rep.*, 1895, 5, p. 379.
[2] *Johns Hopkins Hosp. Bull.*, 1892, 3, p. 17.
[3] *Jour. Exper. Med.*, 1897, 2, p. 197.
[4] *Ibid.*, 1898, 3, p. 611.

in the necrotic areas. Rats were infected by subcutaneous inoculation, and after varying intervals, india-ink was injected into the portal vein. In this way there was readily produced a diffuse and uniform injection of all the liver capillaries. The necrotic areas, however, remained completely uninjected, as shown in Fig. 6,

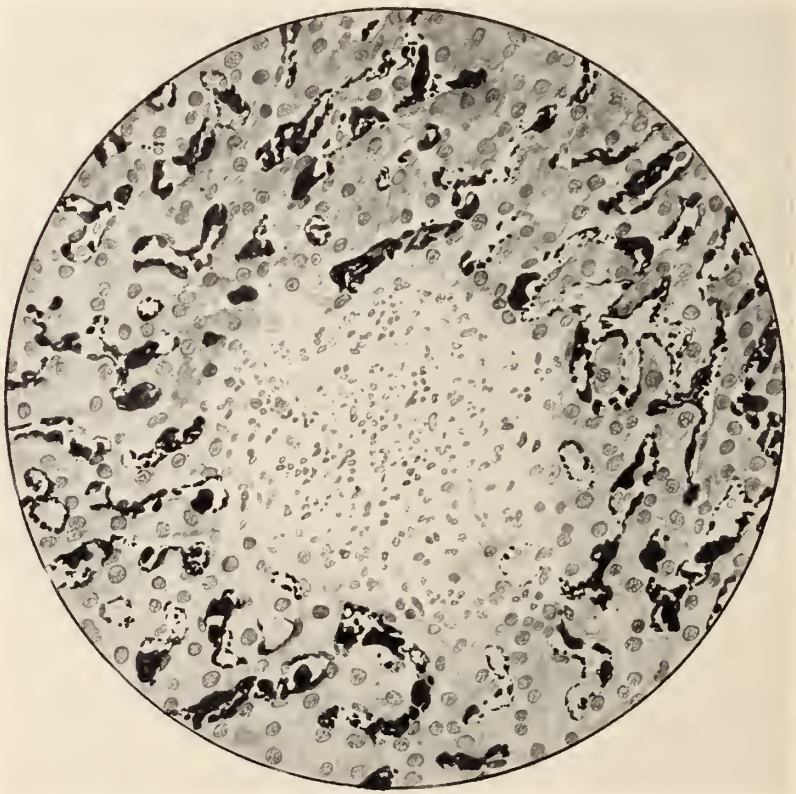

FIG. 6.—Liver of experimentally infected rat after injection of india-ink into portal vein. The necrotic area is impervious to the injection fluid.

and stood out conspicuously from the surrounding injected liver tissue. The capillaries in even the smallest and presumably the earliest necroses could be shown in serial sections to be absolutely impermeable to the injection fluid.

This fact, it seems to us, argues against a primary toxic injury to the epithelial cells, followed by a secondary thrombotic occlusion;

for, in such an event, one would expect to find lesions in which the capillaries were at least partially permeable to the injection fluid.

Because of the profound destruction of cells in the spleen, it seemed probable that the cell fragments found in the liver capillaries might be largely derived from this source. The fact that the intestinal lesions were relatively slight also seemed to bear out this idea. The same view had been previously expressed by Boxmeyer, who, in discussing the source of the large mononuclears found in the liver capillaries, stated that "in the mouse . . . . it is safe to conclude that by far the greater number arise in the spleen and are carried to the capillaries of the liver."

To test this point, we removed the spleens from two rats, infected them, together with controls, and killed them after three days. The liver necroses were of the same character in both sets of animals, and were associated with the formation of fibrin thrombi about cell-fragments. If anything, the necrotic areas were more numerous in the splenectomized rats than in the controls. From these experiments, it would seem that the presence of cells derived from the spleen is not essential for the development of the liver lesions.[1]

That necrotic cells, cell-fragments, and phagocytes are discharged from the spleen and are held back by the capillaries of the liver and spleen could be shown not only by finding such cells in masses in the afferent veins of the liver and in the pulmonary arteries, but by comparing smears made directly from the splenic vein with smears from the left ventricle.

The following count will serve as an example:

Rat M-5-2.—Injected subcutaneously. Killed after 57 hours. Typical gross and microscopic lesions.

Smear from splenic vein:	Per cent
Polymorphonuclear neutrophiles	26
(Of these, 10 showed degeneration, i.e., vacuolization of cytoplasm, loss of granules, indistinct nuclear outline, pale staining of nucleus; some of the leukocytes contained bacilli.)	
Small lymphocytes	11
(Of these, 2 showed distinct pyknosis, with irregularity of nucleus.)	
Large lymphocytes	14
(Of these, 3 showed indistinct nuclear outlines, pale staining, etc.)	

[1] Attempts to ligature the portal vein and thus to prevent the embolism of cells from the intestinal tract as well as the spleen, were unsuccessful, the rats surviving the operation only three to four hours.

	Per cent
Large mononuclear cells	18
(Characterized by round or reniform nucleus with distinct nucleolus, abundant faintly basophilic cytoplasm often studded with purplish granules. Many of these cells inclose one or several bacilli. One cell found undergoing mitosis.)	
Degenerated cells, unclassified	31
(Degenerated cells of various types, some mononuclear, some apparently derived from swollen polynuclears. They often enclose more or less disintegrated bipolar bacilli and occasionally nuclear fragments.)	
Smear from heart blood (left ventricle):	
Polymorphonuclear neutrophiles	34
(Of these, 5 showed degeneration. Several inclosed one or several bacilli, one a red blood cell, one a nuclear fragment of normoblast or lymphocyte.)	
Small lymphocytes	9
Large lymphocytes	31
Large mononuclears (not degenerated)	18
Degenerated cells, unclassified	8

In the smear from the splenic vein, no less than 46 per cent of all the leukocytes showed distinct evidence of degeneration, as against 13 per cent in the blood from the left ventricle. A similar difference was found in other infected rats. Altho the classification of many of the cells found in the smears may be open to question, the contrast is sufficiently striking to indicate that degenerated cells, and particularly phagocytes with bacillary or nuclear inclusions, pass out from the spleen and are held back by the capillaries of the liver and lungs from the general circulation—at least in part.

Since some of the phagocytes contain bacilli, it is difficult to say with certainty that the bacilli or their disintegration products are not concerned in the production of the liver lesions. Even if, as we believe, the lesion begins as a cell-fragment thrombus in the capillaries, it is readily conceivable that the death of the adjacent liver cells is due, not so much to a mechanical obstruction of the circulation, as to the toxic influence of bacilli included in the thrombosed area. Too much emphasis should not be laid upon the failure to find bacilli in the lesions, since the degeneration forms, particularly the intracellular fragments, are extremely difficult to identify with certainty in sections. If, however, identical lesions could be produced by the injection of sterile cellular suspensions into the portal circulation, it would exclude the bacterial factor as essential to the production of the liver lesions. To test this possibility, the spleen was removed aseptically from healthy rats, and a rather dense suspension made by teasing the tissue in sterile

Ringer's solution. This was freed from coarser particles, and, after varying periods, injected through a fine glass pipette, into the mesenteric veins of large rats. The injected animals were killed after intervals varying from three and one-half to 48 hours. The results of these experiments were inconclusive. Distinct lesions were found only in the liver of the rat killed after three and one-half hours. In this animal, they simulated so closely the lesions found in the bacillary infection that the suspicion arose that the rat had been spontaneously infected with the disease at the time of the experiment. Cultures, however, yielded only a few colonies of a white staphylococcus. The other four experiments were wholly negative.

This inconclusive result leads us to express with caution a decided view as to the pathogenesis of the lesions. Certain definite inferences may, however, be drawn from a study of the histological features of the disease. It is plain that the bacterial toxin, whatever its nature, brings about an intense destruction of leukocytes and lymphoid cells in all hematopoietic tissues—spleen, lymph-nodes and bone-marrow. Associated with this injury, there is an active proliferation of large mononuclear cells, many of which become phagocytic and in turn degenerate. In many of the organs, and most strikingly in spleen, liver, and lungs, fibrin thrombi are formed about masses of degenerating cells and cell fragments. In the liver where the structural relations are less complicated than in the spleen and bone-marrow, one may state with assurance that the formation of these cell-fragment fibrin thrombi antedates the destruction of the liver cells, and is the first step in the formation of the necrosis. This point, we believe, is clearly brought out by our ink-injection experiments, as well as by a careful study of the early experimental lesions, in which the thrombi may be found unassociated with necrosis of the liver cells.

Whether the liver cells become affected by the mechanical interference with the blood supply, or whether they degenerate because of the toxic effect of bacilli entrapped in the thrombus, or whether possibly they are poisoned by autolytic products from the impacted cells, it is not at present possible to decide.

We wish to express our thanks to Professor W. G. MacCallum and Professor Augustus Wadsworth for suggestions and criticism in the preparation of this paper.

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PASTEURIZATION IN BOTTLES AND THE PROCESS OF BOTTLING HOT PASTEURIZED MILK.*†

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INTRODUCTION.

The process of heating milk in bottles is by no means a new one, for it probably dates back to the work of Soxhlet[1] from 1886 to 1891. In general, however, the object has been partially or completely to sterilize the milk by the use of high temperatures rather than simply to pasteurize it at low temperatures. While the practice of sterilizing or partially sterilizing milk has been extensively practiced in several countries in Europe, the pasteurization of milk in bottles has not been so common.

It is evident from the report of Gerber and Wieske[2] that pasteurization in bottles has been practiced in certain localities for a considerable period of time. According to these authors, pasteurization in bottles by the process of Gerber,[3] which consists of heating milk in bottles for one hour at 65° C. (149° F.) during which they are agitated, had been practiced in their dairies for 15 years previous to 1903.

In this country milk has been pasteurized directly in bottles at various Strauss Infant Milk stations for several years, but this process has not been used on an extensive commercial scale until within the last 2 years. During the summer of 1910 we began an investigation of the bacteria which survived pasteurization in flasks and of the efficiency of the process. A report of this work has been published in *Bulletin 161*[4] of this Bureau.

While this work was in progress, North[5] suggested the pasteurization of milk in bottles on a commercial scale by the use of machines similar to those which have been in use in breweries for several years.

The process of pasteurizing in bottles consists of bottling the milk in specially constructed bottles of sufficient size to allow a space in the top of the bottle to take care of the expansion of the milk during heating. The bottles are capped with special water-tight caps and submerged in hot water. After the milk in the bottles has reached the pasteurizing temperature, the temperature is maintained for 30 mins.; the hot water is then replaced by cold and the milk cooled. In general it takes about 30 mins. to heat the bottles, 30 mins. for the holding period, and 30 mins. to cool.

This process of pasteurizing in bottles is now used on a commercial scale in a number of milk plants throughout this country.

Numerous advantages of this method of pasteurization over the ordinary methods have been claimed particularly in relation to the far superior bacterial reductions obtained. The most obvious point of advantage of this process is the prevention of

[1] *München. med. Wchnschr.*, 1891, 38, pp. 335 and 353.

[2] *Rev. Gen. du Lait*, 1903, 2, p. 169.

[3] *Loc. cit.*

[4] *Bull. 161, Bureau of Animal Industry, U.S. Dept. Agric.*

[5] *Med. Record*, 1911, 80, p. 111.

reinfection after pasteurizing, but it seems as if a modification of the present system of "holder" pasteurization by bottling the pasteurized milk while hot, as suggested previously by one of us,[1] will help to solve the problem of reinfection.

OBJECT OF THIS INVESTIGATION.

The general object of this work has been to compare on a laboratory scale pasteurization in bottles with the process of bottling hot pasteurized milk. The special objects have been to determine the bacterial reductions in each process, to determine any special points which must be considered in the operation of each process, and to present preliminary data on the cooling of milk in bottles by an air blast.

METHOD OF BACTERIOLOGICAL ANALYSIS.

Since bacterial counts are widely influenced by differences in media and incubation it is always essential in discussing the results of bacteriological work to explain exactly how the counts were obtained. In this work plain infusion agar, made according to the recommendations[2] of the Committee on Milk Analysis, was used. The plates were incubated for 5 days at 30° C. (86° F.) and counted.

METHOD OF PASTEURIZING IN BOTTLES.

Milk was placed in special bottles, similar to those supplied to the trade, and capped with patented metal caps by machine. The bottles were heated by being submerged in hot water at a temperature of from 145° to 147° F. After the temperature in the bottom of the bottles had reached 145° F. they were held at that temperature for 30 mins. and removed, plates being made while the milk was hot. The bottles were so constructed that, after a full quart of milk was poured in, there remained an air space of sufficient size to take care of the expansion during the heating. While heating, it was noticed that the milk expanded and enough pressure was generated to lift the caps slightly so as to allow air to escape. Special care was taken to see that the temperature in the bottom of the bottle of milk was maintained for the full 30 mins.

The method of pasteurization was the same as is used on a commercial scale, hence the results obtained are directly applicable to commercial conditions. The fact that the bacterial counts were taken directly after heating has no effect on the results, since we have shown that cooling plays no part in the destruction of bacteria in the pasteurizing process.[3]

BACTERIAL REDUCTIONS BY PASTEURIZATION IN BOTTLES.

It has been claimed that remarkable bacterial reductions have been obtained by pasteurization in bottles which were far superior to those obtained by other methods even when the same temperature and holding period were used. In order to determine what reductions could be obtained, 34 samples of milk were pasteurized in bottles.

[1] S. H. Ayers, *Circular 184, Bureau of Animal Industry, U.S. Dept. Agric.*

[2] *Am. Jour. Public Hygiene*, 1910, N.S., 6, p. 315.

[3] *Op. cit.*

As may be seen from Table 1, the bacterial reductions were high as a rule but there were exceptions. The average total count of the samples of raw was 1,570,493 and after pasteurization, 9,863 bacteria per cubic centimeter. It is interesting to note that the percentage reductions averaged 90.86 per cent and ranged from 17.67 per cent to 99.98 per cent. When a 99.98 per cent reduction was obtained the raw milk contained 8,100,000 bacteria per cubic centimeter and 28,300 when the reduction was only

TABLE 1.

BACTERIAL REDUCTIONS DURING THE PROCESS OF PASTEURIZATION IN BOTTLES.

Sample Number	Raw Milk	Raw Milk Pasteurized in the Bottle for 30 Mins. at 145° F.	Percentage Reduction
	Bacteria per c.c.	Bacteria per c.c.	
2*	58,000	1,630	97.18
3*	63,000	1,070	98.30
4*	5,100,000	11,800	99.76
5*	580,000	8,000	98.62
6*	5,900,000	15,600	99.74
7*	99,000	980	99.01
8*	7,400,000	7,100	99.90
9*	191,000	7,600	96.02
10*	14,100,000	14,200	99.89
11*	24,700	5,780	75.59
12*	75,000	28,000	62.66
13*	126,000	1,720	98.63
14*	4,100,000	2,410	99.94
15*	76,000	3,550	95.32
16*	8,100,000	1,660	99.98
17*	18,900	710	96.24
18*	24,000	10,900	50.41
19*	28,300	23,300	17.67
20*	80,000	2,010	97.48
21*	160,000	29,500	81.56
22*	151,000	12,500	91.72
23*	81,000	9,800	87.90
24	24,900	570	97.71
25	94,000	2,200	97.66
26	305,000	55,800	81.70
27	235,000	7,600	96.76
28	176,000	11,400	93.52
29	97,000	8,350	91.39
30	230,000	5,500	97.61
31	124,000	1,500	98.79
32	450,000	11,400	97.46
33	3,950,000	3,520	99.91
34	985,000	18,400	98.13
35	190,000	9,300	95.10
Average	1,570,493	9,863	90.86

* The bottles for these samples were washed clean in hot water, but not steamed, before they were filled with raw milk. The bottles for the other samples were steamed 2 mins. and then cooled before they were filled with raw milk.

17.67 per cent. These results further substantiate our conclusion expressed in *Bulletin 161* that percentage bacterial reduction has no special meaning since it is influenced by the number and kinds of bacteria in the milk when pasteurized. Considering the results as a whole, it is evident that low counts may be obtained by pasteurization in bottles.

While carrying on these experiments several points were noted which are worthy of attention.

POINTS TO BE OBSERVED IN THE PROCESS.

Temperature of the milk during heating.—In the process of pasteurization it was found that the temperature of the milk in different parts of the bottle was quite different during the time the milk was being heated. Several experiments were made, heating water in sealed bottles to determine the differences in the top, middle, and bottom of the bottles. Three thermometers were inserted into a bottle through a rubber stopper so that the stems were at the top, middle, and bottom of the bottle, respectively. The bottles were then submerged in hot water at a temperature of from 145° to 146° F. and the temperatures in the bottles of water were recorded. Four pint bottles and 4 quart bottles were used. The averaged temperatures in the pint bottles are shown in Fig. 1. It will be seen from the curves that in a pint bottle with water at 50° F. submerged in hot water at about 145° F. it took $10\frac{4}{5}$ mins. longer for the

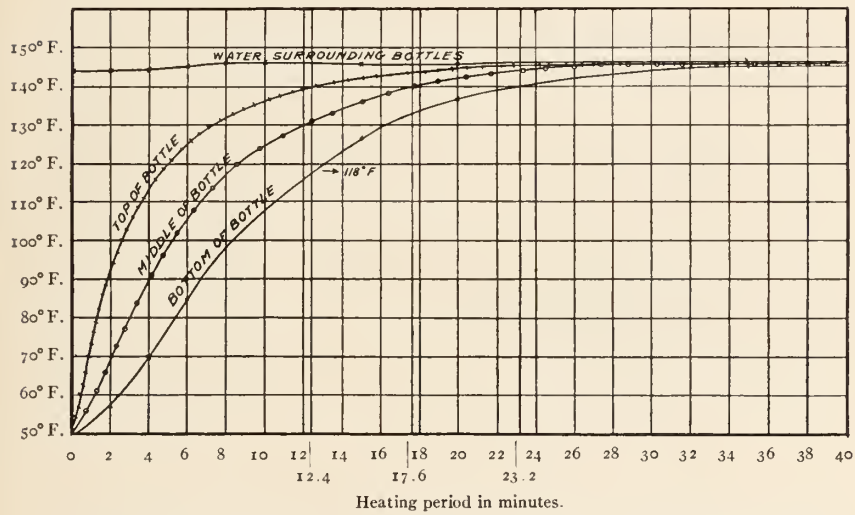

FIG. 1.—Variations in temperature in different parts of pint bottles of water during the process of pasteurization in the bottle.

temperature in the bottom of the bottle to reach 140° F., after the top had reached that temperature and $4\frac{3}{5}$ mins. longer for the temperature in the middle of the bottle. When the temperature in the top of the bottle was 140° F., in the bottom it was only 118° F.

The averaged temperatures of 4 quart bottles are shown in Fig. 2. When the temperature in the top of the bottle was 140° F. that in the bottom was only 127° F. and it took $9\frac{1}{5}$ mins. longer for the temperature in the bottom to reach 140° F.

It is evident that when pasteurizing in the bottle care must be taken to record the temperature in the bottom of a bottle and to date the holding period of 30 mins. from the time the bottom temperature has reached 145° F. In recording the temperature an accurate thermometer should be used and it should reach to within $\frac{1}{2}$ in. of the bottom of the bottle.

Cooling.—After the milk is heated in bottles on a commercial scale it is cooled by replacing the hot water by cold and gradually changing the temperatures so as not to break the bottles. Upon cooling, the hot milk contracts and a partial vacuum is formed in the bottle when the caps are tight. It is recommended by the manufacturers of some of the patent caps that after heating, the bottles be allowed to cool for a few minutes in air until the cap becomes concave, as this is said to hold the cap on tight and helps to make it water-tight. Obviously, it is of utmost importance that the caps be water-tight since they are submerged in water during cooling, and if not tight the milk may become infected by polluted cooling water.

The ordinary cardboard cap is of no value for pasteurization in the bottle since water will easily penetrate during cooling. This makes it necessary to use some form

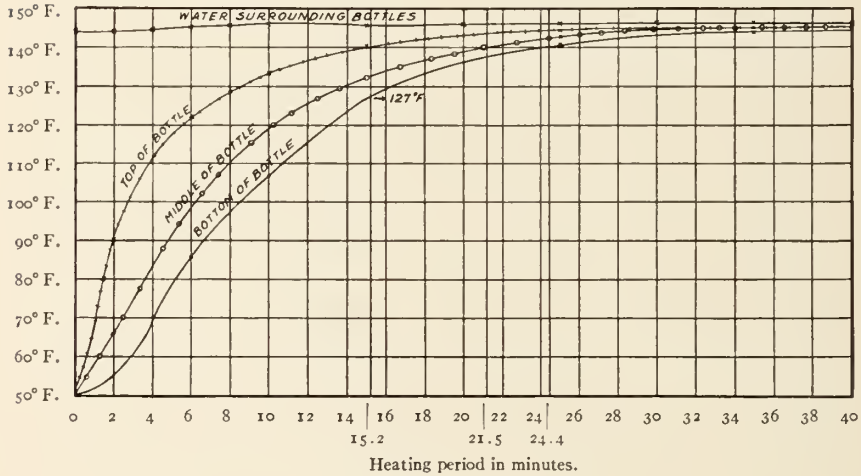

FIG. 2.—Variations in temperature in different parts of quart bottles of water during the process of pasteurization in the bottle.

of patented cap of which both specially treated cardboard and metal caps are on the market. It is almost needless to state that if the edge of the bottle is chipped or otherwise imperfect, almost any seal cap will not be water-tight during the cooling. Imperfect bottles must not be used. It is claimed by the manufacturers of patented seal caps that they are tight on perfect bottles. It would be advisable, however, for the dairyman to test the tightness of his caps by the following method: Fill the milk bottle with a 0.05 per cent solution of barium chlorid ($BaCl_2$). The barium chlorid solution should be made up with distilled water since the sulfates present in ordinary water will cloud the solution. Cap the bottles in the usual way with a seal cap and heat to 145° F., submerge, and cool in a 10 per cent solution of magnesium sulfate ($MgSo_4$).

If any of the magnesium sulfate leaks into the bottle during cooling, the barium chlorid solution will become cloudy owing to the formation of barium sulfate, which is insoluble. This test is very delicate and will show even a slight leak. Both these chemicals may be obtained at any drug-store. *Since barium chlorid is poisonous, after testing bottles in which it has been used care must be taken to wash the bottles thoroughly*

in order to remove the barium solution. Care must also be exercised to keep the chlorid solution from all edible products about the plant.

Having considered these points, let us turn to the advantages and disadvantages of this process.

ADVANTAGES OF PASTEURIZATION IN BOTTLES.

From a bacteriological standpoint the advantage of pasteurization in bottles lies in the fact that reinfection after pasteurization is prevented. There is no chance of the milk becoming contaminated after pasteurization up to the time it reaches the consumer, provided, of course, the seals are water-tight. In the ordinary methods of pasteurization there is a great opportunity for infection from coolers and in bottling. Of course proper handling in the ordinary method of pasteurization reduces and may prevent subsequent reinfection but the chance still remains.

It is the general opinion that the process of pasteurization in bottles produces a great saving in milk by doing away with the loss in evaporation over the coolers and with the loss in milk which adheres to the apparatus in the process of pasteurization. Undoubtedly this saving is quite a considerable factor. There may also be a saving in the expense of machinery and in the interest on the capital invested, but it is not the province of this paper to discuss the financial aspect of this process.

DISADVANTAGES OF PASTEURIZATION IN BOTTLES.

In a plant where pasteurization is now performed it means an entirely new equipment for this system of pasteurization in the bottle. Perhaps the greatest disadvantage is the cost of water-tight caps. This item of expense is important since it may increase the cost of pasteurization as much as one-fifth of a cent per bottle. Whether the saving in milk losses is sufficient to overcome this added expense can be determined only by the actual operation of a milk plant.

METHOD OF PASTEURIZING MILK IN BULK AND BOTTLING WHILE HOT.

For the pasteurization of milk in bulk a double-walled cylindrical tin tank with a capacity of about 3.5 gals. was used. The construction of this tank is shown in Fig. 3. Raw milk was placed in the milk-tank where it was heated by hot water in the outer jacket. The surrounding water was heated by a steam jet and constantly

agitated by blowing in a small amount of air. During the heating the milk was agitated by a paddle supported by the cover of the milk tank. The water in the jacket was kept at a temperature of about 146° F. The milk was held at a temperature of 145° F. for 30 mins. and then drawn off while hot through the outlet pipe into hot milk bottles which had been steamed 2 mins. As stated before, this method of bottling

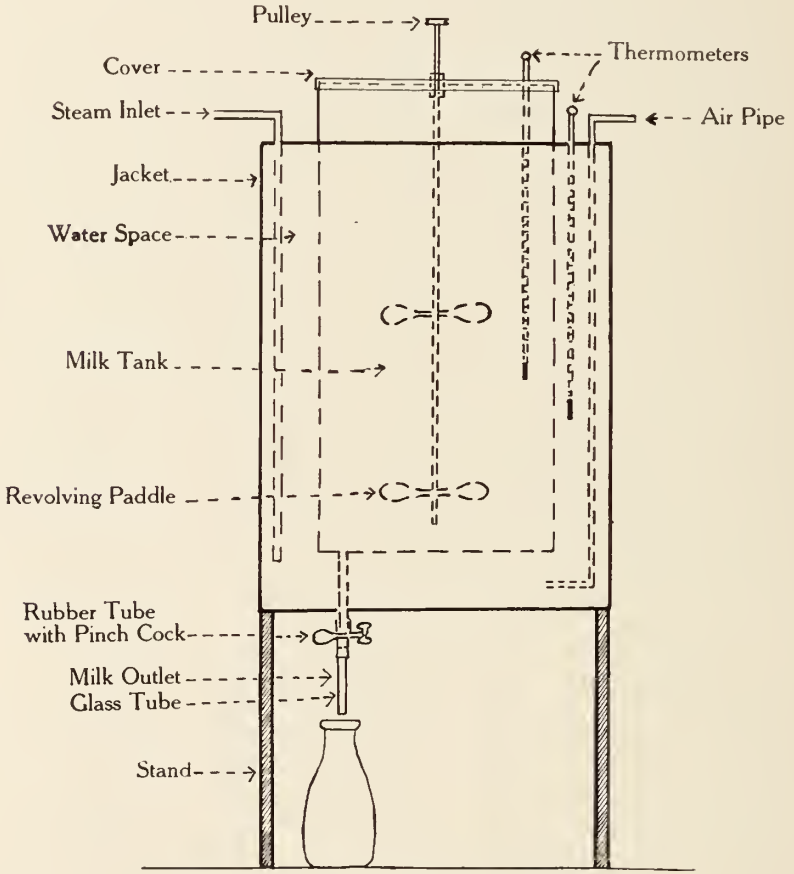

FIG. 3.—Apparatus for pasteurizing milk in bulk.

milk while hot was suggested in *Circular 184*,[1] but the suggestion then was to bottle hot milk in cold bottles. In this work it seemed advisable to bottle directly into hot bottles as it makes it possible to steam the bottles and fill them before infection can take place. Also, this method eliminates the possibility of breaking bottles. While working on this process of bottling milk hot it has been found that a similar process was apparently patented several years ago but, so far as we are aware, it has never

[1] *Loc. cit.*

been used to any extent. This process as described by De Schweinitz[1] consisted in pasteurizing the milk at temperatures from 160° to 180° F. and placing it while hot in a sterilized milk jar or fruit jar with a flat top. Special paper caps were used. The jars of milk were cooled by being placed in troughs of iced water.

COMPARISON OF BACTERIAL REDUCTIONS IN MILK PASTEURIZED IN BOTTLES AND BY THE PROCESS OF BOTTLING MILK WHILE HOT.

Since it has been shown earlier in this paper that excellent bacterial reductions could be obtained by pasteurization in bottles, a question of great importance arises as to whether or not as good results can be obtained by pasteurizing milk in bulk and bottling while hot.

A series of 22 samples of raw milk were pasteurized by both processes at 145° F. for 30 mins. Part of the milk was pasteurized in bulk in the pasteurizer shown in Fig. 3, and bottled hot in hot bottles which had been previously steamed for 2 mins. In all

TABLE 2.

COMPARISON OF BACTERIAL REDUCTIONS IN THE PROCESS OF PASTEURIZATION IN UNSTEAMED BOTTLES AND BOTTLING HOT PASTEURIZED MILK.

SAMPLE NUMBER	RAW MILK	MILK PASTEURIZED AT 145° F. FOR 30 MINS.			
		Hot Pasteurized Milk in Hot Steamed Bottle		Milk Pasteurized in Bottles*	
	Bacteria per c.c.	Bacteria per c.c.	Percentage Reduction	Bacteria per c.c.	Percentage Reduction
2	58,000	1,160	98.00	1,630	97.18
3	63,000	220	99.65	1,070	98.30
4	5,100,000	8,400	99.83	11,800	99.76
5	580,000	8,300	98.57	8,000	98.62
6	5,900,000	6,000	99.90	15,600	99.74
7	99,000	610	99.38	980	99.01
8	7,400,000	6,300	99.91	7,100	99.90
9	191,000	2,000	98.95	7,600	96.02
10	14,100,000	7,000	99.95	14,200	99.89
11	24,700	4,550	81.58	5,780	75.59
12	75,000	3,000	96.00	28,000	62.66
13	126,000	1,440	98.86	1,720	98.63
14	4,100,000	2,470	99.94	2,410	99.94
15	76,000	1,400	98.16	3,550	95.32
16	8,100,000	1,620	99.98	1,660	99.98
17	18,900	760	95.97	710	96.24
18	24,000	800	96.66	10,900	50.41
19	28,300	7,050	75.09	23,300	17.67
20	80,000	1,360	98.30	2,010	97.48
21	160,000	1,830	98.86	29,500	81.56
22	151,000	3,200	97.88	12,500	91.72
23	81,000	6,800	91.60	9,800	87.90
Average	2,115,268	3,467	96.50	9,083	88.34

* Bottles were washed clean in hot water, but not steamed, before they were filled with raw milk.

[1] *Yearbook, U.S. Dept. Agric.*, 1894, p. 33.

these experiments bottles were capped with ordinary paper caps, no precautions being used in capping by hand. Another portion of the same raw milk was pasteurized in bottles. Both samples of pasteurized milk were examined bacteriologically while hot in the bottles.

In the first series the bottles in which the milk was pasteurized directly were washed in hot water and washing powder immediately before they were filled with raw milk.

The results of bacteriological examinations are shown in Table 2. It will be seen that the average count of the raw milk was 2,115,268 bacteria per cubic centimeter. After being pasteurized in bulk and bottled hot in hot steamed bottles the average count was 3,467 bacteria per cubic centimeter, while the average count of some of the same milk pasteurized in bottles was 9,083 bacteria per cubic centimeter. Comparing the percentage of bacterial reductions, it will be noted that the average reduction of the milk bottled hot was 96.50 per cent and only 88.34 per cent in the milk pasteurized in bottles. In 19 of the 22 samples the bacterial count was lower in milk pasteurized in bulk and bottled hot. In many cases the count was much lower, as may be seen by comparing Samples 4, 6, 7, 12, and 18. This difference is particularly striking in Sample 21 in which milk pasteurized in bulk and bottled hot showed a count of 1,830 and some of the same milk pasteurized in a bottle for the same time and at the same temperature contained 29,500 bacteria per cubic centimeter.

In the belief that this marked difference might be due to the fact that when milk was bottled hot the bottles were steamed for 2 mins. and when the milk was pasteurized directly in bottles they were only washed in hot water, another series of samples were pasteurized in which both bottles were steamed for 2 mins. in order to eliminate this factor of possible infection. The results of these experiments are shown in Table 3.

Similar results, however, were obtained in this series of 12 samples in which 10 showed lower counts when pasteurized in bulk and bottled hot.

The average count of the raw milk was 571,766 bacteria per cubic centimeter. After pasteurization in bulk followed by bottling

hot the count was 5,965, and some of the same milk pasteurized in bottles averaged 11,295 bacteria per cubic centimeter. In several of the samples the count in milk pasteurized in bottles was very much higher than the same milk pasteurized in bulk and bottled hot. We are at a loss to explain these marked differences. While minor differences are always within the limits of the errors of bacteriological methods, the great differences found in many cases cannot be explained in this manner.

TABLE 3.

COMPARISON OF BACTERIAL REDUCTIONS IN THE PROCESS OF PASTEURIZATION IN STEAMED BOTTLES AND BOTTLING HOT PASTEURIZED MILK.

SAMPLE NUMBER	RAW MILK	MILK PASTEURIZED AT 145° F. FOR 30 MINS.			
		Hot Milk in Hot Steamed Bottle		Milk Pasteurized in Bottles*	
	Bacteria per c.c.	Bacteria per c.c.	Percentage Reduction	Bacteria per c.c.	Percentage Reduction
24	24,900	380	98.47	570	97.71
25	94,000	860	99.08	2,200	97.66
26	305,000	21,800	92.85	55,800	81.70
27	235,000	5,400	97.70	7,600	96.76
28	176,000	2,200	98.75	11,400	93.52
29	97,000	5,900	93.91	8,350	91.39
30	230,000	6,300	97.26	5,500	97.61
31	124,000	920	99.26	1,500	98.79
32	450,000	4,200	97.47	11,400	97.46
33	3,950,000	4,320	99.89	3,520	99.91
34	985,000	11,800	98.80	18,400	98.13
35	190,000	7,500	96.06	9,300	95.10
Average	571,766	5,965	97.46	11,295	95.48

* Bottles were steamed 2 mins. and cooled before they were filled with raw milk.

THE PREVENTION OF INFECTION BY THE PROCESS OF BOTTLING MILK WHILE HOT AND BY PASTEURIZATION IN BOTTLES.

Since the process of pasteurizing milk in bulk and bottling hot enables the use of hot, steamed bottles which can be directly filled with hot milk, it should be expected that there would be no contamination added to the milk during bottling.

To determine this point 8 samples of milk were pasteurized in bulk and bottled hot in hot, steamed bottles. The bacteriological results are shown in Table 4. Two steamed and cooled milk bottles for each sample were inoculated with equal amounts of sour milk. One of these infected bottles was then steamed for 2 mins. and filled with hot pasteurized milk and the other contaminated bottle not heated was filled with some of the same pasteurized milk which had been previously cooled in a sterile bottle. An examination of Table 4 shows, when the figures in Columns A and C are

compared, that the infectious material added to the bottle was entirely destroyed by the method of bottling, at least as far as bacteriological methods can detect, since any marked increase in Column C would show infection. Column B shows the bacterial counts obtained by putting cold pasteurized milk in infected bottles. From these results it is evident that the process of bottling hot pasteurized milk in hot, steamed (2 mins.) bottles entirely eliminates the factor of bottle infection which may often be serious in the ordinary processes of pasteurization on a commercial scale.

TABLE 4.

DESTRUCTION OF BOTTLE INFECTION DURING THE PROCESS OF BOTTLING HOT PASTEURIZED MILK.

Sample Number	Raw Milk	Hot Pasteurized Milk in Hot Steamed Bottles	Cold Pasteurized Milk in a Cold Bottle†	Hot Pasteurized Milk in a Steamed Bottle†
	Bacteria per c.c.	Bacteria per c.c.	Bacteria per c.c.	Bacteria per c.c.
24	24,900	380	6,400,000	460
25	94,000	860	5,600,000	600
27*	235,000	5,400	1,330,000	4,800
28	176,000	2,200	1,510,000	2,400
29*	97,000	5,900	235,000	4,100
30*	230,000	6,300	355,000	5,800
31*	124,000	920	305,000	950
35*	190,000	7,500		8,800
		A	B	C

* Bottle infected with old sour pasteurized milk.

† Bottles had been previously infected with several cubic centimeters of sour milk.

TABLE 5.

DESTRUCTION OF BOTTLE INFECTION DURING THE PROCESS OF PASTEURIZATION IN BOTTLES.

Sample Number	Raw Milk	Milk Pasteurized in Clean Previously Steamed Bottle	Bottle Infected with Several Cubic Centimeters of Sour Milk and Filled with Raw Milk	Milk Pasteurized in an Infected Bottle
	Bacteria per c.c.	Bacteria per c.c.	Bacteria per c.c.	Bacteria per c.c.
24	24,900	570	3,700,000	2,090
25	94,000	2,200	3,300,000	6,200
27*	235,000	7,600	760,000	9,500
28	176,000	11,400	650,000	11,000
29*	97,000	8,350	530,000	20,000
30*	230,000	5,500	645,000	20,900
31*	124,000	1,500	400,000	28,600
35*	190,000	9,300	230,000	9,600
36*	38,000	5,600	92,000	17,700
		A	B	C

* Bottle infected with old sour pasteurized milk.

The question naturally arose as to whether or not pasteurization in bottles would destroy infection in bottles specially infected before being filled with raw milk. To determine this point 9 samples of milk were pasteurized in bottles which had been previously steamed and cooled. The results are shown in Table 5. One bottle for each sample was steamed, cooled, infected with several cubic centimeters of sour milk, and filled with some of the original raw milk. Samples were then plated from this bottle to show the extent of the infection, the results of which may be found in Column B of the table. The bottle of infected raw milk was capped with a seal cap and the

milk pasteurized directly in the bottle. Plates were made directly after the heating and the bacteriological results are shown in Column C. Any increase in the counts in Column C over those in Column A shows the amount of infection introduced by placing milk in an infected bottle.

It is evident that only in 2 samples, 28 and 35, was the infection entirely destroyed.

It is quite possible that infection from unclean bottles might become a serious factor in bottle pasteurization. When one considers that in pasteurization in the bottle the bacteria which are left are either heat-resistant vegetative cells or spores, it is easy to see that if a large number are left in a bottle and it is again filled with milk and pasteurization again performed in the bottle these same bacteria will again survive and increase the number left. It is advisable to steam the bottles at least 2 mins. before filling with milk for pasteurization in the bottles.

COOLING MILK WHICH HAS BEEN BOTTLED HOT.

Use of an air blast.—It is of course possible to bottle the milk while hot, cap with a water-tight seal, and cool by submerging in cold water, but we have been experimenting with a process by which the milk may be cooled in bottles capped with ordinary cardboard caps. Briefly stated, the process consists in exposing the hot bottled milk to an air blast. The air blast system is used at present in hardening rooms in ice cream plants but, as far as we are aware, this system has never been applied to the cooling of milk.

Several experiments were tried on a laboratory scale which gave promising results. When a bottle of hot milk is allowed to cool in still air a film of warm air forms about it which can move away only by convection and, naturally, the cooling process is slow. If some means were provided for moving the film of warm air and forcing cool air against the bottle, heat would constantly be given up with more rapidity by the milk and the cooling process hastened. In Fig. 4 are shown the temperatures in 3 bottles of milk cooled for 30 mins. in air. One bottle was cooled in still air at 77° F., one was cooled in an air blast from an electric fan at a temperature of 77° F., and one was cooled in still air at 35° F. At the beginning of the cooling the temperature of the milk was about 145° F. As will be seen from the curves after 30 minutes' cooling, the temperature of the milk in the bottle cooled in still air at 77° F. was about 127.5° F., while that of the milk cooled in an air blast at 77° F. was only about 102° F. It is noted that by cooling in an air blast for 30 mins. there was a reduction in temperature of about 25.5° F. in excess of that obtained under the same conditions in still air. The temperature curve of the milk in the bottle cooled in still air at 35° F. follows closely that of the milk cooled in still air at 77° F. It is also interesting to note that after cooling for 30 mins. in still air at 35° F. the temperature was 122° F. while that of the milk cooled in an air blast at 77° F. was about 102° F., a difference of 20° F.

Since these experiments indicated that hot bottled milk might be cooled more rapidly by using a blast of cold air, another experiment was conducted in which one quart and one pint bottle were cooled in still air which averaged 39.4° F. and another set in an air blast the temperature of which averaged 44.3° F. The blast of cold air was obtained by placing an electric fan in a refrigerator. The fan delivered air at a velocity of about 1,250 ft. per minute. The temperature curves in Fig. 5 show the results of this experiment. The temperatures of the hot milk at the beginning of the cooling ranged from 140° to about 143.5° F. in the different bottles. It will be seen from

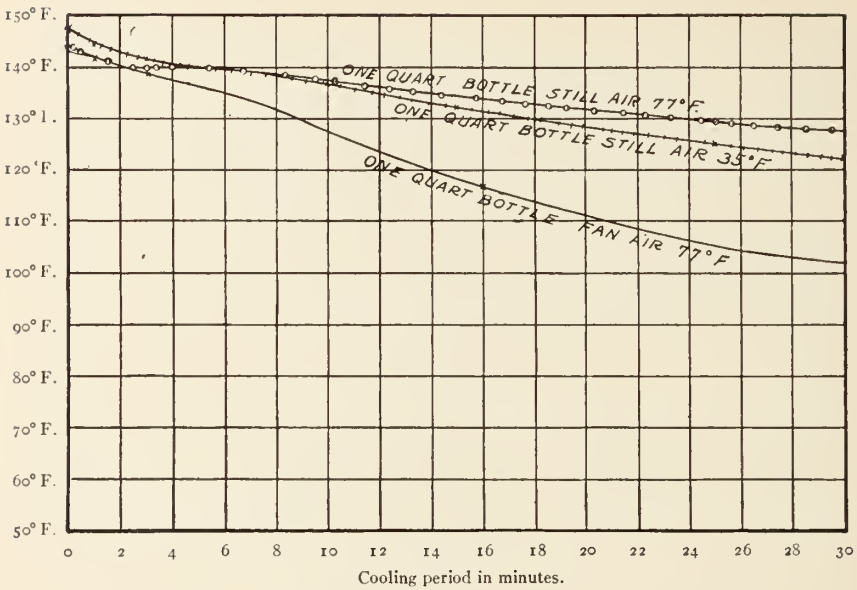

FIG. 4.—Effect of cooling a quart bottle of milk in still air and in an air blast.

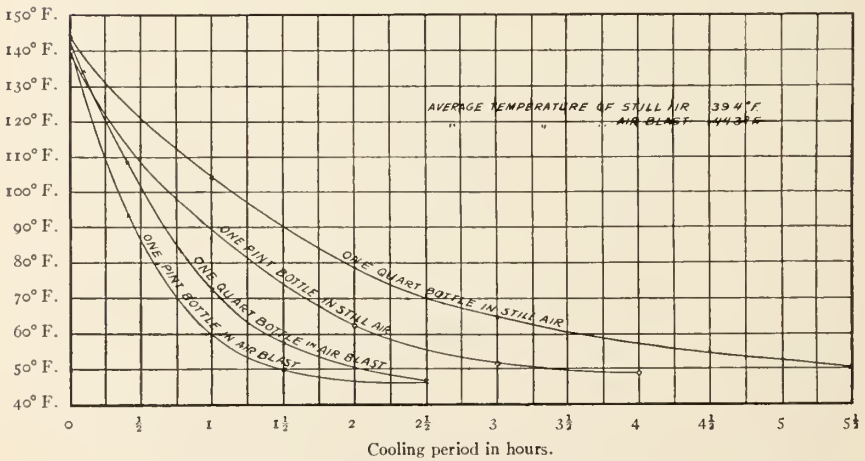

FIG. 5.—The cooling of pint and quart bottles of hot milk in still air and in an air blast at refrigerator temperature.

the curves that $5\frac{1}{2}$ hrs. were required for the temperature of the quart bottle of milk in still air to reach 50° F., while the milk in a quart bottle in an air blast was cooled to 50° F. in a little over 2 hrs. The milk in the pint bottle cooled in still air, reached a temperature of 50° F. after about $3\frac{1}{2}$ hrs., while only $1\frac{1}{2}$ hrs. were required to cool the milk in the pint bottle which was in a blast of cold air.

From these results there can be no doubt as to the value of an air blast for cooling bottles of hot milk, at least over still air as a cooling medium. As these experiments were made on single bottles it was thought advisable to try cooling several crates of bottled hot milk by an air blast. Specially constructed skeleton frame steel crates were used so as to allow a free circulation of air.[1] Milk was pasteurized at 145° F. for 30 mins. and bottled hot in ordinary milk bottles by the aid of a hand bottle-filler.

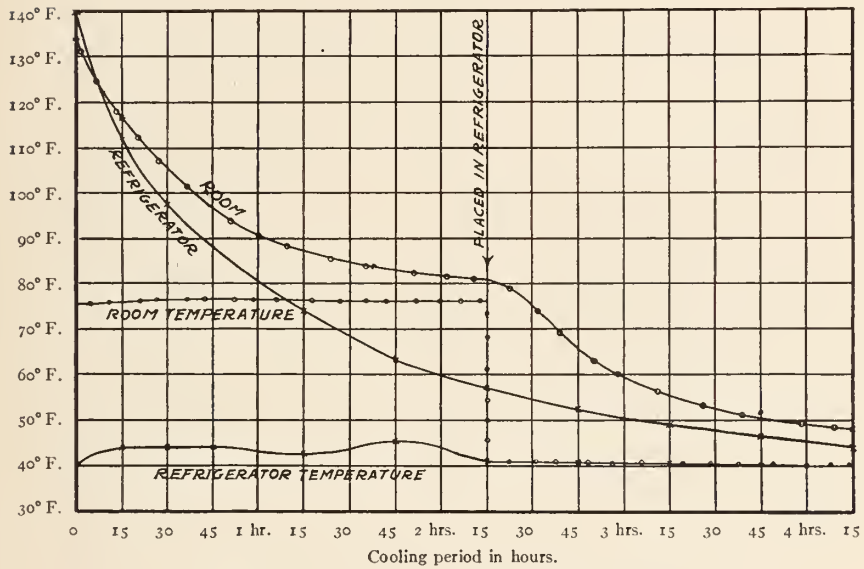

FIG. 6.—Effect of cooling bottled hot milk in an air blast at different temperatures.

The bottles were then capped with the ordinary cardboard caps and placed in crates. Four crates were used in these experiments, 2 filled with quart and 2 with pint bottles. The 2 crates which contained quart bottles were placed in a refrigerator room one above the other and directly back of them were placed the 2 crates of pint bottles one above the other. The air blast was generated by a 16-inch desk fan which gave an air velocity of about 1,250 ft. per minute. The fan was placed about $2\frac{1}{2}$ ft. in front of the pile of 4 crates directly facing the crates with quart bottles. Temperatures were taken in 2 quart bottles, one in the front and the other in the back row. In this experiment the crates were cooled in a refrigerator room the temperature of which varied from 40° to 44° F. The results of this experiment are shown in curves in Fig. 6, together with the results of a similar experiment in which the crates were cooled in an

[1] In this work we were assisted by John T. Bowen of this division.

air blast at a temperature of about 76° F. for a period of 2¼ hrs. The crates were then placed in a refrigerator and the cooling continued, a blast of air with a temperature of about 41° F. being used. The curves in Fig. 6 show the averaged temperatures of 2 quart bottles. It will be seen from Curve A that about 3 hrs. and 7 mins. were required to cool the milk in quart bottles from 140° to 50° F. when cooled in a blast of cold air during the entire period. A comparison of Curves A and B shows that it took only about 45 mins. longer to cool to 50° F. the milk in bottles exposed to an air blast at room temperature for the first 2¼ hrs. It is interesting to note that curves A and B follow each other fairly closely during the first 30 mins. of cooling. These results suggest that the cooling of hot pasteurized bottled milk may be accomplished by cooling with an air blast at ordinary room temperature and completed by cooling in a blast of cold air in a refrigerator room. The greater the number of heat units which can be removed from the milk by an air blast at room temperature the cheaper the cost of cooling, since refrigeration would be saved and about the only cost would be the operation of a blower.

These experiments, altho by no means conclusive as to the value of this method of cooling by an air blast on a practical scale since many complications may arise in the practical application, indicate great possibilities for such a system.

The effect of slow cooling after pasteurization on the bacterial flora of milk.—We are aware that any system of pasteurization in which the milk is not cooled immediately after heating will be looked upon with suspicion and will excite comment. It has always been supposed that immediate cooling was an indispensable part of the process of pasteurization, (1) because sudden changes in temperature were believed to have a destructive effect on the bacterial cells, and (2) because it has been supposed that bacteria left after pasteurization would immediately begin to grow unless the milk was cooled at once.

As stated earlier in this paper, we have shown in *Bulletin 161*[1] that sudden cooling played no part in the destruction of bacteria. There remains, therefore, one question to be answered: How quickly must pasteurized milk be cooled in order to check bacterial growth?

From our former studies of pasteurization it seemed apparent that the bacteria which survived heating were somewhat weakened or at least did not begin to grow as might theoretically be expected. These observations naturally gave rise to the idea that pasteurized milk might be cooled directly in bottles by a cold air blast provided the cooling period would not extend over a few hours.

[1] *Loc. cit.*

In order to obtain data on this question 10 samples of milk were pasteurized and bottled hot in steamed bottles. One bottle was cooled within half an hour in ice water and placed in a refrigerator at 45° F. for 17½ hrs. In each case the other bottle was cooled slowly at room temperature for 4 hrs. and placed in a refrigerator at 45° F. for 14 hrs. At the end of that time each bottle of milk was 18 hrs. old; one was cooled quickly and had been at 45° for 17½ hrs. The other had been cooled slowly and had been at 45° for probably a very short time, because altho it had been in the refrigerator for 14 hrs., the milk was warm when placed there and cooling in still air is a slow process. Both bottles after the 18-hr. cooling period were allowed to stand at temperatures from 75° to 86° F. for a period of 6 hrs.

TABLE 6.

BACTERIAL GROWTH DURING A QUICK AND SLOW COOLING TO 50° F. AND IN SUBSEQUENT HOLDING FOR 6 HRS. AT WARM TEMPERATURES.

Bacteria in 1 c.c.			Sample Number									
			1	2	3	4	5	6	7	8	9	10
	Raw milk		95,000	176,000	176,000	97,500	97,500	.....	450,000	.....	985,000	38,000
	Milk Cooled Quickly	Directly after pasteurization	600	1,870	1,570	5,900	5,900	22,900	890	4,800	8,300	5,500
		After 18 hrs. held at 45° F.	1,000	2,050*	2,370*	.....	.....	16,600	1,700	2,500	8,900	5,200
		Sample then placed for 6 hrs. at 86° F.	.....	5,750	8,400	6,600	5,900	.....	.....	.....	9,600†	5,200†
	Milk Cooled Slowly	Directly after pasteurization	860	1,320	1,220	5,900	5,900	21,800	890	5,400	7,500	6,500
		Cooled slowly at room temperature for 4 hrs. and placed for 14 hrs. in refrigerator at 45° F.	500	1,180	5,520	.....	.....	12,300	2,200	715	9,800	5,200
		Then placed for 6 hrs. at 86° F.	.....	5,800	6,100	3,700	3,700	.....	.....	.....	8,900†	5,300†

* Held at 45° F. for 21 hrs. in place of 18 hrs. † Held at 75° F. instead of 86° F.

As may be seen from Table 6, bacterial counts were made of the raw milk on each bottle directly after pasteurization, at the end of the 18-hr. cooling period, and again after the milk had been at room temperature for 6 hrs. The bacterial results obtained showed that there was no more increase in the pasteurized milk cooled slowly than in similar milk cooled within half an hour and held at low temperatures for 18 hrs. Neither was there any difference in the bacterial numbers even after milk cooled by both processes had been removed, after 18 hours' cooling, and allowed to stand for 6 hrs. The various counts from 10 samples have been averaged and are given in Table 7 in order to show more plainly the effect of the two systems of cooling on the bacterial numbers in milk. It will be seen from Table 7 that the average bacterial counts at different times of the milk cooled slowly are even lower than those of milk cooled quickly. While this difference is probably an experimental error, it is evident that bacterial growth in the pasteurized milk was not increased by the slow cooling process.

We do not wish to convey the idea that pasteurized milk need not be cooled at all. The cooling of any milk is absolutely essential in order to restrain bacterial growth, and we wish to emphasize the fact that the process of cooling pasteurized milk slowly does not dismiss the cooling process but simply makes use of a slower cooling process than is in use at present.

TABLE 7.

Summary of Results from Table 6.

	Milk	Bacteria per c.c.
Cooled quickly	Directly after pasteurization	5,823
	After 18 hrs. in refrigerator	5,040
	Taken from refrigerator and held 6 hrs. at temperatures from 75° to 86° F	6,908
Cooled slowly	Directly after pasteurization	5,729
	Cooled slowly at room temperature for 4 hrs. and held in refrigerator for 14 hrs. at 45° F	4,678
	Taken from refrigerator and held for 6 hrs. at temperatures from 75° to 86° F	5,583
Raw milk		264,375

TABLE 8.

Effect of Different Methods of Cooling on the Bacterial Content of Pasteurized Milk.

			Sample Number		
			1	2	3
Bacteria in 1 c.c. of milk	Raw milk		9,050,000		11,900,000
	Cooled Quickly	Directly after pasteurization	6,450	2,110	8,500
		Held at 45° F. for 22 hrs	5,050	1,720	28,400
		Held at 75° F. for 6 hrs	4,800	2,340	76,500
		Held at 75° F. for 24 hrs	1,370,000	885,000	
	Cooled Slowly	Directly after pasteurization	7,150	2,580	11,900
		Held at 75° F. for 5 hrs	6,100	1,600	29,000
		Held at 45° F. for 17 hrs	6,200	2,400	192,000
		Held at 75° F. for 6 hrs	9,600	2,740	348,000
		Held at 75° F. for 24 hrs	2,760,000	850,000	
	Allowed to Cool Naturally in Air 75° F.	Directly after pasteurization	4,950	2,180	8,500
		Held at 75° F. for 5 hrs	6,850	2,890	25,900
		Held at 75° F. for 22 hrs	700,000	2,420,000	83,400,000
		Held at 75° F. for 28 hrs	2,750,000	13,400,000	269,000,000
		Held at 75° F. for total of 66 hrs	460,800,000		

In order to show, respectively, the effect of cooling quickly, cooling slowly, and not cooling to low temperatures at all, 3 experiments were made. Milk was pasteurized in bulk and 3 steamed and hot quart bottles were filled with hot milk. One bottle was cooled in iced water in half an hour to 50° F. and refrigerated at 45° F. Another bottle was cooled in a blast of air at room temperature for half an hour during which time the temperature dropped from 145° to about 100° F. The milk was then allowed to stand at a temperature of from 100° to 80° F. for 5 hrs., after which it was placed in a refrigerator at 45° F. where it cooled slowly in still air. The remaining bottle was cooled for half an hour in an air blast at room temperature and allowed to remain at a temperature of about 75° F. through the entire experiment. The results of these

experiments, in which bacterial counts were made at different stages of the cooling process, are given in Table 8. A study of the table shows that there was no increased bacterial growth in Experiments 1 and 2 caused by holding the pasteurized milk for 5 hrs. after bottling hot, even tho the temperature during that period ranged from 100° to 80° F., which is the most favorable temperature for bacterial development. In Experiment 3 there was an increased growth over that in the milk cooled quickly. It must be remembered that these experiments represent extreme conditions in slow cooling, but the fact is apparent that the cooling process should not extend over 5 hrs. The effect of not cooling milk to low temperatures is plainly shown in the table by a comparison of the bacterial counts with those of milk cooled both quickly and slowly. We believe from these experiments that it is possible to cool hot bottled pasteurized milk by a blast at room temperature followed by a blast of cold air without any more bacterial development than would take place if the milk were immediately cooled, provided the milk is cooled to 50° F. gradually within 5 hrs. This is not made as a definite statement because different results may, of course, be obtained when milk is thus cooled on a commercial scale.

Again we wish to emphasize the fact that pasteurized milk or raw milk must be kept at low temperatures after cooling in order to check bacterial development.

EFFECT OF THE PROCESS OF BOTTLING HOT PASTEURIZED MILK FOLLOWED BY SLOW COOLING ON THE CREAM-LINE AND FLAVOR OF MILK.

In the consideration of the process of bottling hot pasteurized milk followed by slow cooling it is of practical importance to know what effect such a process will have on the cream-line and flavor of milk. Several experiments were made to determine the effect on these points. Milk was pasteurized and hot 500-c.c. graduated cylinders were filled with hot milk up to the 500-c.c. mark. Together with the cylinder of hot pasteurized milk one cylinder was filled with raw milk and one with pasteurized milk which had been cooled to 50° F. in 15 seconds' time by running through a coil immersed in brine. The method of cooling the hot, bottled, pasteurized milk was varied considerably, as may be seen from Table 9. After holding the milk for 24 hrs. at 45° F. the numbers of cubic centimeters of cream were read off directly from the graduations on the cylinder. This method, of course, gave a very accurate means of determining the effect of heating and cooling on the cream-line, and in fact it was too accurate since considerable differences in the cream-line by this method of measurement were not apparent in bottled milk. A study of the results in Table 9 shows that cream-line formation is a variable factor. Sometimes it was reduced by pasteurization

even when the milk was cooled to low temperatures within 15 secs. and at other times there was no difference. In some experiments the cream-line was slightly less on milk cooled slowly and again it was slightly higher. Throughout our experiments on pasteurized

TABLE 9.

CREAM-LINE EXPERIMENTS.

Experiment Number	Milk	Process	Cubic Centimeters of Cream in 500 c.c. Cylinder after 24 hours' Refrigeration at 45° F.
1	Raw milk		64.5
	Milk pasteurized at 145° F. for 30 mins.	Cooled quickly in 15 secs. to 50° F. and held in refrigerator at 45° F.	64.5
		Cooled slowly in air blast for 45 mins. and placed in refrigerator at 45° F.	65.0
		Held above 105° F. for 3 hrs., cooled in ice water, and placed in refrigerator at 45° F.	64.5
2	Raw milk		65.0
	Milk pasteurized as above	Cooled in 15 secs. to 50° F. and placed in refrigerator at 45° F.	62.5
		Cooled slowly in air blast for 1½ hrs. and placed in refrigerator at 45° F.	52.5
		Held above 100° F. for 1½ hrs. and placed in refrigerator at 45° F.	52.5
3	Raw milk lost		
	Milk pasteurized as above	Cooled in 15 secs. to 50° F. and placed in refrigerator at 45° F.	83
		Cooled slowly for 30 mins. in air blast, cooled quickly in brine, and placed in refrigerator at 45° F.	85
		Held above 100° F. for 3 hrs., cooled quickly in brine, and placed in refrigerator at 45° F.	90
4	Raw milk lost		
	Milk pasteurized as above	Cooled in 15 secs. to 50° F., and placed in refrigerator at 45° F.	75
		Cooled slowly in air blast for 2½ hrs., cooled in ice water, and placed in refrigerator at 45° F.	69
		Held above 100° F. for 2½ hrs., cooled in ice water, and placed in refrigerator at 45° F.	75
5	Raw milk		80
	Milk pasteurized as above	Cooled in 15 secs. to 50° F. and placed in refrigerator at 45° F.	68
		Cooled slowly in air blast for 2 hrs. and placed in refrigerator at 45° F.	55
		After cooling in air blast for 2 hrs. the milk was cooled quickly in brine to 50° F. and placed in refrigerator at 45° F.	62
		Held above 100° F. for 5 hrs. and placed in refrigerator at 45° F.	55
		After holding above 100° F. for 5 hrs. the milk was cooled quickly in brine to 50° F. and placed in refrigerator at 45° F.	62

milk bottled hot in ordinary milk bottles a good clear cream-line was obtained. When milk stood at temperatures above 80° F. for several hours without agitation some of the melted butter fat rose to the top of the bottle and on cooling formed a small lump of

butter. This was not observed, however, when the cooling process was begun immediately after bottling even tho the cooling was gradual.

As to the effect on the flavor of the milk, it may be said that there was no more effect than that produced by milk pasteurized and cooled rapidly except in instances where the milk was held above 100° F. for several hours as was the case in some of our experiments, in which a slightly more pronounced cooked taste was noticeable in the milk.

In this connection we wish to call attention to the fact that these results hold only for milk pasteurized at 145° F. and cannot be applied where higher temperatures might be used as it is possible that with higher temperatures different results might be obtained.

BOTTLES TO BE USED WITH THE PROCESS OF BOTTLING HOT PASTEURIZED MILK.

It is obvious that a quart bottle filled with milk at 145° F. will not contain a full quart when the milk has cooled to 50° F., owing to the contraction during cooling. Several experiments which were made to determine the loss in volume during cooling showed a shrinkage in a quart bottle of an average of about 18.40 c.c. Assuming a quart of milk to be 946.35 c.c., that volume at 145° F. contracts to about 927.9 c.c. when cooled to 50° F., which is a difference of about 18.40 c.c. If a quart bottle is filled with milk at 145° F. it will be 18.40 c.c. or 0.62 of an ounce short of one quart when cooled to 50° F. To overcome this shortage, bottles of a slightly larger capacity should be used when filled with milk at 145° F. A bottle should be of sufficient size to hold one quart of milk measured at 50° F. which has been heated to 145° F.

AN OUTLINE OF THE PROCESS OF BOTTLING HOT PASTEURIZED MILK.

Having discussed the various steps in the process of bottling hot pasteurized milk, let us outline the possible application of this process to commercial conditions.

Milk is pasteurized by the ordinary holder system at 145° F. for 30 mins. It is then bottled hot in special oversize milk bottles of the ordinary type and capped with the ordinary sterile cardboard

caps. Before being filled, the bottles are steamed for 2 mins. by running the crates inverted on a conveyor over steam jets. The bottles go to the bottling machine in a hot condition and are practically sterile. Skeleton steel crates are used to allow a free circulation of air during cooling. Hot bottles of hot pasteurized milk are placed in a refrigerator room and cooled by a blast of cold air. Blowers may be used in the refrigerating room to produce the air blast. It is also possible to cool the bottles by placing them in an air blast outside of the refrigerator until the temperature reaches 100° F. and then placing them in the refrigerator for the cooling to be continued by a cold air blast until the temperature reaches 50° F In the winter season it might be possible to use cold outside air for cooling the milk at no expense for refrigeration other than the operation of a blower.

Only future work on a commercial scale will determine the true value of the process of bottling hot pasteurized milk.

ADVANTAGES OF THE PROCESS OF BOTTLING HOT PASTEURIZED MILK.

Since the process has not as yet been worked out for practical use it is impossible to state definitely all its advantages and disadvantages. However, from laboratory experiments alone certain advantages are plainly shown. From a sanitary standpoint one great advantage of the process of bottling hot lies in the fact that hot pasteurized milk may be bottled in hot bottles so that bottle infection is eliminated. From a commercial standpoint there is an advantage in the reduction of milk losses on the cooler caused by adherence of milk and by evaporation. Ordinary cardboard caps may be used in this system since they do not have to be watertight; this is obviously a point of great advantage as far as cost is concerned.

At the present stage of this work it is impossible to state how the cost of air cooling will compare with the ordinary methods in practice but we believe that there will be no more expense involved.

DISADVANTAGES OF THE PROCESS OF BOTTLING HOT PASTEURIZED MILK.

There is one disadvantage in this process which may, however, be only theoretical, and that is, that infection might be introduced during capping. Sterile caps are used in the best plants and are put on by machine, hence the danger from this source may not be important. Since the milk is hot when capped another factor is introduced to prevent infection from this source. The length of time required for cooling is perhaps the greatest disadvantage of this process and yet this would be of no consequence except in plants where milk is delivered immediately after pasteurization. In the majority of milk plants milk is pasteurized in the morning or afternoon, placed in refrigerators, and delivered early the next morning. Consequently, in most plants it would make little difference whether the cooling process was performed quickly or slowly.

SUMMARY.

1. The process of pasteurization in the bottle using a temperature of 145° F. for 30 mins. causes satisfactory bacterial reductions.

2. Bottles should be steamed before being filled with milk for at least 2 mins. in order to destroy heat-resistant types of organisms which might survive the pasteurizing temperature and thereby increase the bacterial count.

3. Care must be taken to record the temperature in the bottom of the bottle during the heating process. When milk at an initial temperature of 50° F. is heated in bottles without agitation in water at about 146° F. the temperature of the milk in the top of the bottle will reach 140° F. about 9 mins. before that in the bottom. The temperature of the milk during the process of pasteurizing in the bottle should be recorded by placing a thermometer in a control bottle with the bulb of the thermometer about $\frac{1}{2}$ in. from the bottom. The milk should be heated for 30 mins. at 145° F.

4. Care should be taken not to use bottles with chipped or otherwise imperfect tops since the seal caps may allow leaks during the process of pasteurizing. It is advisable for the users of patented seal caps to assure themselves that the caps are water-tight since

leaking caps might cause dangerous infections particularly if the cooling water is polluted.

5. The great advantage of the process of pasteurization in bottles over the ordinary systems in use at present lies in the fact that no recontamination can take place from the time of pasteurization until it reaches the consumer, when the caps are water-tight. There is also a saving in milk losses due to handling and evaporation over coolers which is usual in the ordinary methods of pasteurization.

Considerable expense, however, is incurred owing to the necessity of water-tight caps, which is a disadvantage of the process. Whether the saving in milk losses makes up for the additional expense of seal caps can be determined only by experience.

6. The process of bottling pasteurized milk while hot into hot steamed bottles causes equally good bacterial reductions as does pasteurization in bottles. Even with the same lengths of exposure of 30 mins. and the same temperature of 145° F. the bacterial reductions are often much greater than those produced by pasteurization in bottles.

7. In the process of bottling hot, bottle infection is eliminated even when several cubic centimeters of old sour milk are added to bottles before filling. The 2-mins. steaming period to which the bottles are subjected before filling with hot milk is sufficient to destroy the contamination at least so far as bacteriological methods can detect.

8. Laboratory experiments indicate that milk may be pasteurized, bottled hot, capped with ordinary cardboard caps, and cooled by a blast of cold air.

9. It is probable that if milk is cooled from 145° to 50° F. within 5 hrs. no more bacterial increase will take place during the slow cooling than would take place if the milk were cooled immediately to 50° F. Whether or not this will be true under commercial conditions can be determined only by future experiments.

10. As far as our laboratory experiments indicate, when milk is heated to 145° F. for 30 mins., bottling hot pasteurized milk followed by slow gradual cooling has no more appreciable effect on the cream-line or flavor of milk than does the ordinary process

of pasteurization. This is true for cooling periods of less than 5 hours' duration.

11. Since milk contracts on cooling, a quart bottle filled with milk at 145° F. does not hold a full quart when the milk is cooled to 50° F. It is about 0.62 of an ounce short. Therefore slightly oversized bottles should be used.

12. The advantages of the process are: (1) that bottle infection can be eliminated, (2) that milk losses are saved, owing to evaporation over the cooler, and (3) that ordinary cardboard caps can be used. The principle disadvantage is that the air-cooling process requires several hours. This, however, would be a disadvantage only in the few plants where milk is delivered directly after pasteurization.

CONCLUSIONS.

In conclusion we wish to state that the process of bottling hot pasteurized milk followed by air-blast cooling appears from our laboratory experiments to be an entirely feasible modification of the "holder" system of pasteurization. It must be remembered, however, that this system has not been applied commercially and we are aware that many unforeseen complications may arise.

In view of these facts this process will be the subject of a future study on a commercial scale in which the details of bottling and air-blast cooling can be worked out in a manner which will prove or disprove the practical value of this process.

FERMENTS FOR CARCINOMATOUS PROTEIN IN THE BLOOD IN CARCINOMA.*

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By means of the method of dialysis devised by Abderhalden, Brockmann,[1] Frank and Heimann,[2] and Epstein[3] have found that the serum of the blood in cases of carcinoma contains ferments which act on the proteins of carcinoma tissue. Such ferments were not found in the serum of persons free from carcinoma.

I have made an attempt to estimate quantitatively the amount of proteolysis in carcinomatous material when acted on by carcinomatous and normal serum under comparable conditions. At first the optical method was used. In this case the carcinoma tissue ("antigen") was prepared by grinding with sand and extracting with salt solution over night in the icebox. The extract was centrifugated and the fluid passed through Berkefeld filters and heated to 60° C. for 10–15 minutes in order to destroy the ferments in the material itself. Of such extracts 10 c.c. were mixed with 2 c.c. of serum and the optical rotation determined at once and after incubation for 3 hrs. at 37° C. The results follow:

	1. CARCINOMA OF UTERUS.		2. CARCINOMA OF UTERUS.		3. CARCINOMA OF BREAST.	
	Patient's Serum	Normal Serum	Patient's Serum	Normal Serum	Patient's Serum	Norma Serum
At once.....	50′	53′	35′	40′	43′	40′
After incubation	45′	54′	31′	39′	39′	39′
	5′	1′	4′	1′	4′	1′

In these three tests there is evidence of some proteolysis by the carcinomatous serum and of less by the normal serum, altho the differences in the normal readings are not beyond the limits of unavoidable error.

The preparations of the material for the optical method being difficult, other tests were made by the method of titration of amino

* Received for publication January 26, 1914.

[1] *Lancet*, 1913, 185, p. 1384.

[2] *Berl. klin. Wchnschr.*, 1913, 50, p. 631.

[3] *Wien. klin. Wchnschr.*, 1913, 26, p. 650.

acids (Henriques, Sorensen). The carcinomatous tissue was prepared as before but the extracts were not filtered. For each complete test about 5 gm. of tumor were used made up into 40 c.c. of extract, 20 c.c. of which were mixed with 4 c.c. of carcinomatous serum and 20 c.c. with 4 c.c. of normal serum. To each mixture were added 3 c.c. of toluene, with thorough shaking to prevent bacterial growth. The amounts of amino acids in 10 c.c. of each mixture were now determined in terms of $\frac{2}{10}$ sodium hydrate before and after incubating for 24 hrs., the difference in the amount of amino acids in the 2 parts of the mixtures representing the amount of proteolysis taking place during the incubation. The 10 c.c. tested before incubation were removed by means of a pipette as soon as the toluene formed a layer at the top. In each test the 10 c.c. of serum mixture were measured into a 200 c.c. Erlenmeyer flask; to this, 100 c.c. of distilled water and 5 drops of 0.5 per cent solution of phenolphthalein were added and then N/10 NaOH until a faint pink tinge appeared. Next 5 c.c. of formalin were diluted to 25 c.c. with distilled water and 5 drops of 0.5 per cent solution of phenolphthalein added. The solution was brought to a faint pink color by adding N/10 NaOH. The neutral formalin was then added to the neutral carcinoma-serum mixture and the resulting acidity measured by a burette reading of the amount of N/10 NaOH necessary to restore the pink color. The mixtures of carcinoma extract and serum were made at icebox temperature so as to prevent as much action as possible before the first amino acid titration.

The results of the experiments are given in terms of N/10 NaOH.

	4. Carcinoma of Uterus.		5. Carcinoma of Uterus.		6. Carcinoma of Uterus with Omental Metastasis; Extract from Metastasis.	
	Patient's Serum	Normal Serum	Patient's Serum	Normal Serum	Patient's Serum	
1st titration....	2 c.c.	2.1 c.c.	3.40 c.c.	3.4 c.c.	1.05 c.c.	No Control
2d "	2.5 "	2.2 "	3.95 "	3.9 "	0.95 "	
	0.5 "	0.1 "	0.55 "	0.5 "	0.1 "	

	7. Recurrent Carcinoma of Uterus.		8. Carcinoma of Uterus.		9. Carcinoma of Uterus.		
	Patient's Serum	Normal Serum	Patient's Serum	Normal Serum	Patient's Serum	Serum of Case 10.—Carcinoma of Uterus	Normal Serum
1st titration..	2.25 c.c.	2.25 c.c.	4.95 c.c.	4.75 c.c.	2.20 c.c.	2.4 c.c.	2.4 c.c.
2d " ..	2.20 "	2.30 "	4.30 "	5.15 "	2.05 "	2.9 "	2.9 "
	0.05 "	0.05 "	0.65 "	0.60 "	0.75 "	0.5 "	0.5 "

Inasmuch as there was a well-marked change from the normal in Case 9 a comparison was made between that serum and the serum of Case 10. Instead of extract of uterine carcinoma an extract from a carcinoma of the breast (Case 3) was used to see if there would be the same difference in this case also. The action of all 3 sera was tried also on a 2 per cent gelatin peptone solution at the same time to find out whether or not a difference existed between the action of carcinoma serum on carcinoma and other protein material. The results follow:

Extract of Carcinoma of Breast.			Gelatin-Peptone Solution.		
Serum of Case 9.—Carcinoma of Uterus.	Serum of Case 10.—Carcinoma of Uterus.	Normal Serum	Serum 9	Serum 10	Normal Serum
2.95 c.c.	3.1 c.c.	2.95 c.c.	0.95 c.c.	1.00 c.c.	1.00 c.c.
3.45 "	5.0 "	3.25 "	1.00 "	1.05 "	1.05 "
0.55 "	1.9 "	0.30 "	0.05 "	0.05 "	0.05 "

From these figures it will be seen that in Case 9 there was a difference from the normal of 0.55 c.c. which would indicate a slightly lower proteolytic on breast carcinoma than on the homologous carcinoma, but in Case 10 there was a difference from the normal of 1.9 c.c., indicating that more proteolysis occurred in the breast carcinoma than in the uterine carcinoma. Experiments with gelatin, on the other hand, showed an entire lack of proteolysis by all 3 sera.

It has been shown[1] that proteolytic processes occur during hemolysis by immune serum and that a certain parallelism exists between the proteolytic power of the blood in pneumonia and the complement content of the blood. Hence, simultaneously with the comparison of the proteolytic power of the blood in the experiments described, a comparison of the complement content of the sera was made. In some cases this was impossible on account of the presence of an amboceptor for the corpuscles used (sheep). The comparisons in the complement content of the blood were made by adding graded amounts of normal and carcinoma sera to constant quantities (0.2 c.c.) of a 5 per cent suspension of sheep corpuscles with an excess of amboceptor equal to 10 times the minimum quantity necessary to hemolyze 0.2 c.c. of corpuscles in the presence of 0.01 c.c. of normal human serum.

[1] Dick, *Jour. Infect. Dis.*, 1911, 9, p. 282, and 1912, 10, p. 383.

The complement of the different specimens of serum is given below in terms of the number of times the strength of normal serum taken as one. Case 1 was 5 times the normal in complement content; Case 2, 1; Case 3, $1\frac{2}{3}$; Case 4, $1\frac{2}{3}$; Case 8, $1\frac{2}{3}$; Case 10, 3. In 3 cases in which the proteolytic power was not estimated the complement was $2\frac{1}{2}$, 1, and $1\frac{2}{3}$ times that of normal serum. It will be seen that as a rule the complement content of the blood in carcinoma is higher than that of normal serum.

SUMMARY.

By the titration method of estimating amino acids, ferments capable of splitting protein from carcinomatous tissue are demonstrable in the blood serum of patients with carcinoma.

The power of the blood serum of carcinomatous patients to split carcinomatous protein does not differ from the normal with the same constancy and to the same degree when estimated by amino acid titration as indicated by the results obtained with the dialysis method of Abderhalden.

Serum capable of splitting protein from carcinoma of the uterus was also capable of splitting protein from carcinoma of the breast, but not mixtures of gelatin and Witte's peptone.

The complement content of the blood serum of a carcinoma patient is as a rule higher than normal.

SOME PHENOMENA INVOLVED IN THE LIFE HISTORY OF SPIROCHAETA SUIS.*†

STUDIES ON HOG CHOLERA.

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The recognition of *Spirochaeta suis*[1] in certain lesions found in cases of hog cholera, and the results attending experimental cultivation and animal inoculation have afforded many interesting observations relative to the life history of the organism. These studies have not yet progressed to such a point that definite conclusions can be drawn as to the cycle through which this organism may pass. However, its morphological variations under different conditions appear to correspond in many respects with those of other spirochetes which have been studied and reported upon by many investigators.[2]

A future publication, which we hope will contribute toward the interpretation of the life cycle of spirochetes in general, will contain more detailed results of our observations of *Spirochaeta suis*, and its morphological and biological variations. This preliminary report is submitted in order that the results of certain filtration experiments may be placed on record.

Dujardin-Beaumetz[3] in discussing *Die Peripneumonie der Rinder* refers to certain interesting filtration experiments as follows:

* Received for publication January 19, 1914.

† Owing to the provisional use of the name "Spirochaeta suis" by Bosanquet (*Spirochetes*, Saunders, 1911) and Neveu-Lemaire (*Parasitologie des Animaux Domestiques*, Paris, 1912) in referring to the findings of Dodd (*Jour. Comp. Path.*, 1906, 19, p. 216) and *Cleland* (*Parasitology*, 1908, 1, p. 218), it will be necessary to designate this organism by another name. This will be done in future publications.

[1] King and Baeslack, *Jour. Infect. Dis.*, 1913, 12, p. 307; King, Baeslack, and Hoffmann, *Jour. Infect. Dis.*, 1913, 12, p. 365; King and Hoffmann, *Jour. Infect. Dis.*, 1913, 13, p. 463.

[2] Gleitsmann, *Centralbl. f. Bakteriol.*, I, *Orig.*, 1913, 68, p. 31; Mühlens, *Handbuch d. Path. Protozoen* Prowazek, Leipzig, 1912, p. 361; Ross, *Brit. Med. Jour.*, 1912, p. 1651; McDonagh, *Lancet, Proc. Roy. Soc.*, 1913, 6, p. 86; Dutton, *Jour. Trop. Med.*, 1907, 10, p. 385; Mayer, *Arch. f. Schiffs.- u. Tropenhyg.*, 1908, 12, *Beihefte* 1, p. 1; Mackinnon, *Parasitology*, 1909, 2, p. 267; Bosanquet, *Spirochetes*, Saunders, 1911; Marchoux and Couvy, *Ann. de l'Inst. Past.*, 1913, 27, p. 450; Meirowski, *München. med. Wchnschr.*, 1913, 60, pp. 1870 and 2042. Fry, Ranken, and Plimmer, *Jour. Royal Army Med. Corps*, 1913, 21, p. 137; Balfour, *Centralbl. f. Bakteriol.*, I, *Orig.*, 1913, 70, p. 182; O'Farrell and Balfour, *Centralbl. f. Bakteriol.*, I, *Ref.*, 1913, 59, p. 292; Noguchi, *Jour. Exper. Med.*, 1912, 16, p. 199.

[3] *Handbuch d. Path. Mikroorg.*, 1913, 8, p. 943.

"ISOLATION OF BACTERIA BY FILTRATION."

"In investigations of pleuropneumonia, various writers have often filtered the pleuropneumonia exudate through 'Gips' and porcelain filters and observed that such filtrate is incapable of causing either infection or immunity in the animals. From this fact it was learned that the pleuropneumonia was not caused by a soluble virus, but by a living organism. But when Löffler had proved that it was possible to infect animals with lymph obtained from aphthous pustules, diluted with water in proportion 1:50 and then filtered, and that there were therefore bacteria which were so infinitely small that they would pass through the filter, the investigations on pleuropneumonia were again taken up, along the lines where positive results were obtained by means of the dilution method. The method of dilution, as well as the quality of the filter, is of the greatest importance for the success of the experiment. If pleuropneumonia exudate or a culture in bouillon, which contains more that 15 per cent of serum, is used for filtration without being previously diluted, failure is usually the result, since the pores of the filter become clogged with the albumen. The choice of candle is not without significance as the different filters possess different degrees of porosity. . . . . The passage of this organism through certain filtering walls is of the greatest interest from the standpoint of bacteriologic technic. The filter, which until now has been regarded as a perfect process for the sterilization of warmed fluid media, can also be of great service in the isolation of very small bacteria, by retaining the other larger microbes in their walls. It might also be possible to isolate and compare the filtrable viruses serially, using for this a set of suitably chosen filters of decreasing porosity.

"If the possibility is also offered of obtaining the pleuropneumonia virus in pure state, it is always wise to inoculate cultures in serum bouillon. If the presence of foreign bacteria in the inoculation material is feared, a preliminary culture on serum agar can be made; this, however, is not advisable. Pleuropneumonia develops very slowly on solid media, and the colonies are not visible until after 3 days' standing at 37°. During this time the other more rapidly developing bacteria, such as the pleuropneumonia bacillus of Arloing, overgrow the surface of the agar, and render isolation of the pleuropneumonia organism impossible. By employment of the filtration method this obstacle is overcome. The fluid can be collected without any special precautions. It can be taken even in the stage of decomposition and still be capable, by filtration, of isolating the organism in pure state. The process is as follows: The lung lymph is diluted in proportion of $\frac{1}{2}$:100 with peptone bouillon containing no serum. The bouillon must be filtered through a porcelain filter, not for purpose of sterilization, but to eliminate all solid particles which are suspended in the fluid and could hinder filtration. The lymph diluted in the above manner is then filtered through Berkefeld or Chamberland filters L_2. It is recommended that the bouillon be first warmed at 37–39°, for it is known that warm fluids can pass through porous strata better, and the passage of the virus is thereby guaranteed. For the certainty of the growth of the specific cause, serum must be added to the filtrate so obtained, in the proportion of 10 parts serum to 100 parts bouillon. To prevent accidental contamination, it is well to filter the serum through the same filter as is used for the filtration of the bouillon. The filtrate, mixed with serum, is placed in the incubator, and after 3–4 days an opalescence is observed in the cultures. If such an opalescence also appears in those tubes which were further inoculated with material from these

cultures, and if no such change can be observed in the control tubes which were inoculated at the same time, the growth of the pleuropneumonia virus is proved, and the costly animal experiment is unnecessary. It is sufficient if only a trace of the opalizing fluid is spread on the serum agar. Very fine colonies, at first transparent, later wartlike, appear on this medium in so characteristic a form as to banish any doubt as to their identity.

"This method can be used not only for the purpose of isolation, but also for veterinary diagnosis in the investigation of lung material obtained from cattle which have succumbed to suspected pneumonia."

In a former publication[1] data were presented showing that the Berkefeld filtrates of cultures containing *Spirochaeta suis* were capable of producing hog cholera in susceptible hogs. In the meantime successful attempts have been made to develop cultures of the spirochete from the Berkefeld filtrates. This work, of which brief notes are given, was undertaken chiefly for the purpose of securing data bearing on three important points: (1) to determine the correlation between the "filtrable virus," or "ultramicroscopic organism" of hog cholera, and *Spirochaeta suis;* (2) to secure pure cultures of the spirochete; and (3) to determine the importance of the granules assumed to be related to *Spirochaeta suis*.

September 23, 1913: A small portion of necrotic tissue containing numerous *Spirochaetae suis*, from ear of Hog 653, was shaken in sterile water and filtered through Berkefeld filter N, pressure less than one atmosphere, time of filtration about 20 mins., and volume of filtrate about 10 c.c. Cultures from the filtrate were made in broth, grown aerobically, and found to be sterile.

Special media, inoculated with the filtrate and incubated under anaerobic conditions, were placed in a desiccator, *in vacuo*, with pyrogallic acid. The media consisted of (1) Hata medium,[2] with hog blood corpuscles, and (2) Hata medium with rabbit kidney tissue and 0.5 per cent phenol.

Dark-Field Examinations.

October 18, 1913: One culture of each medium showed many granules but no spirochetes.

October 31, 1913: One culture of each showed slight contamination with a bacillus, no spirochetes, no granules noted.

November 19, 1913: Two cultures of each showed some contamination, bacillus.

December 9, 1913: Three cultures of each, same as on November 19.

December 15, 1913: Cultures examined, contaminating bacillus found in all. *Spirochaeta suis* found in 2 cultures of Hata medium, rabbit kidney, and 3 cultures of Hata medium, hog corpuscles.

Spirochetes were not numerous, but were found with little difficulty, and some were actively motile.

[1] *Jour. Infect. Dis.*, 1913, 13, p. 484.

[2] Hata, paper read at Seventeenth International Congress of Medical Sciences, London, August, 1913.

December 17, 1913: One of contaminated cultures of *Spirochaeta suis*, examined under date of December 15, 1913, shaken in sterile water and filtered through Berkefeld. The filtrate was inoculated into plain broth, agar slant, and deep glucose agar, and after incubation under both aerobic and anaerobic conditions, no growth resulted.

December 19, 1913: Special media—(1) Hata medium with hog kidney, (2) hog serum medium, with and without hog kidney, and (3) pyrogallic serum medium (Proca, Danila, and Stroe[1]) with hog kidney—were inoculated with Berkefeld filtrate of original culture, No. 653, *Spirochaeta suis*.

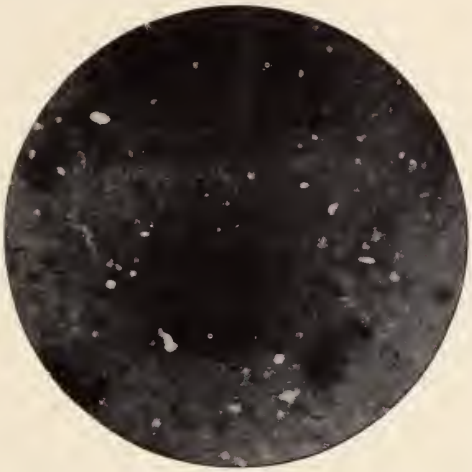

FIG. 1.—Photomicrograph of *Spirochaeta suis* in culture from Berkefeld filtrate of suspension of material, ear lesion, Hog 653. ×1200. (India ink preparation.)

DARK-FIELD EXAMINATIONS.

December 21, 1913: One culture of each medium examined. Contaminating organisms (bacilli and cocci) found. No spirochetes present.

December 24, 1913: Two cultures of each medium examined, same result as on December 21, 1913.

December 29, 1913: Three cultures of each medium examined, same result as on December 24, 1913. As all cultures in this lot appeared to be contaminated, they were discarded. From the check culture tests made, it was evident that contamination with bacilli and cocci was present in the special culture media. Repeated results on other occasions confirm the conclusion that contamination of these special media occurs as the result of adding the kidney tissue and hog corpuscles to the medium after it is prepared.

January 5, 1914: Culture 653, containing *Spirochaeta suis*, first generation, described under first section of these notes, was shaken in sterile water and passed through Berkefeld filter as above. Filtrate was found to contain no organisms capable of developing on broth, agar, or deep glucose agar culture media, when incubated aerobically and anaerobically for several days.

[1] *Compt. rend. Soc. de biol.*, 1912, 72, p. 895.

New lot of special deep media, some containing no kidney tissue or blood clot, was inoculated with filtrate, sterile except for granules presumably related to *Spirochaeta suis*.

DARK-FIELD EXAMINATIONS.

January 9, 1914: Two cultures examined, nothing detected except numerous granules in one culture.

January 12, 1914: Several cultures examined. *Spirochaeta suis* found in two cultures. Not actively motile, small, consisting of about 2–3 convolutions (old cultures show spirochetes of 4–10 convolutions, average number, perhaps 6).

January 15, 1914: Third culture found to contain spirochetes, morphology similar to those noted on January 12, 1913.

From the above short series of experiments no definite conclusions should be drawn. One must recognize the fact that filtration experiments are difficult to control and many erroneous results have been recorded in the literature from time to time.

The results of the experiments outlined in this paper have been controlled by logical methods, and are suggestive of the theory that *Spirochaeta suis*, at some period in its life cycle, is capable of passing through bacteria-proof filters.

A NON-CHOLERA VIBRIO RESEMBLING THE TRUE CHOLERA VIBRIO AND A PIGMENT-FORMING VIBRIO.*

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A NON-CHOLERA VIBRIO.

Since Koch in 1883 isolated the true cholera vibrio other vibrios have at different times been isolated. Some of these have borne a marked resemblance in many respects to the Koch vibrio, but have failed to answer to all the requirements, failing always in the agglutination and bacteriolytic tests and some in being hemolytic and possessing more than one flagellum. In some of these cases in observing the marked similarity the question has arisen whether these vibrios may not be the cholera vibrio under different conditions of growth, which have caused it to lose its pathogenicity and its power to react to the tests; but it is generally conceded that such is not the case and that these vibrios are not related to the true cholera vibrio.

This vibrio, Case 7050 of the series of steerage passengers examined for cholera vibrios during the summer and the autumn of 1911, is reported on account of its similarity to the true cholera vibrio. It was isolated from the rectal contents of an Italian, 22 years old, who arrived November 15, 1911. After this vibrio, which so closely resembled the true cholera vibrio, was found in his rectal contents he was removed to Swinburne Island Hospital for further observation. The preliminary examination showed this small vibrio to be present in large numbers. It did not agglutinate with the cholera serum in any dilution whatever. On November 21 it was still present in the stools, but on November 27 and December 10 and 11 it had completely disappeared.

This vibrio gave a moist, white growth on alkalin agar, the colony being larger, moister, and slimier than that of cholera. It had the typical morphology and motility of the true cholera vibrio. An alkalin peptone culture gave a surface growth and a faint indol

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TABLE 1.

A NON-CHOLERA VIBRIO.

Name	Source	Morphology	Gram	Motility	Colony	Gelatin	Indol	Peptone	Flagellum	Pathogenicity	Agglutination with Cholera Serum
True cholera vibrio.....	Human feces	Small curved rod	Negative	Very rapid	White, opalescent, becoming granular	Rapidly liquefied	Cholera red	Pellicle aerobe	One	For man	Positive
Spirillum of Metchnikovi.	Feces and blood of domestic fowl	Small curved rod	Negative	Very rapid	Larger	More rapid	Cholera red	Pellicle aerobe	One	Fowls and guinea-pigs	Negative
Spirillum of Massowah ..	Human feces	Small curved rod	Negative	Very rapid	.....	More rapid	.....	Pellicle aerobe	Four	Birds	Negative
Spirillum of Finkler and Prior.....	Human feces	Thicker in the center and more pointed at the ends	Negative	Very rapid	.....	More rapid	Negative	Pellicle aerobe	One	.....	Negative
Spirillum of Deneke.....	Cheese and butter	Much like that of Finkler and Prior	Negative	Very rapid	.....	More rapid	Negative	Pellicle aerobe	One	.....	Negative
No. 7050.....	Human feces	Like cholera vibrio	Negative	Very rapid	Larger, moister, and slimier	More rapid	A faint red	Pellicle aerobe	One	.....	Negative

reaction; gelatin was rapidly liquefied; and acid was produced in dextrose and saccharose peptone but not in lactose. It was strongly hemolytic on blood agar. It possessed but one flagellum, in this respect resembling the true cholera vibrio and that of Metchnikovi, Finkler and Prior, and Deneke. Inoculated twice intraperitoneally into guinea-pigs it produced no pathogenic results.

Table 1 will show the points of similarity and dissimilarity between the true cholera vibrio and a few of the other vibrios, which in many respects bear a striking resemblance to it. The points of similarity between the Koch vibrio and No. 7050 are (1) the source, human feces; (2) the morphology, a small curved rod; (3) staining, gram-negative; (4) motility, very rapid and darting; (5) gelatin, rapidly liquefied; (6) peptone,

aerobic growth; and (7) flagellum, one. The points of dissimilarity are (1) the colony, which in the case of No. 7050 is larger, moister, and slimier than that of the true cholera vibrio; (2) the faint cholera red it gives in contrast to the marked red in the case of the latter; and (3) its pathogenicity and the negative reaction with the cholera agglutinating serum. No. 7050 differs from the spirillum of Metchnikovi (1) in having been found in the human feces while the latter was isolated from the feces and the blood of the domestic fowl and also (2) in the pathogenicity of the latter for fowls and guinea-pigs. It differs from the spirillum of Massowah in having only one flagellum while the latter has four. Its morphology differs from that of the vibrio of Finkler and Prior in that the latter is thicker in the middle and more pointed at the ends. It also differs in this respect from the spirillum of Deneke, which is, however, found in cheese and butter and not in the human feces. It bears closer resemblance to the vibrio of true cholera than to any of the others here mentioned, which have a human source, i.e., the spirillum of Massowah and

TABLE 2.

A Pigment-forming Vibrio.

Name	Source	Morphology	Motility	Stain	Colony	Gelatin	Peptone	Indol	Hemolysis	Pathogenicity	Agglutination with Cholera Serum
True cholera vibrio.....	Human feces	Small curved rod	Very rapid and darting	Gram-negative	White and opalescent, becoming coarsely granular	Rapidly liquefied	White surface growth, aerobic	Positive	Negative	Man	Positive
Pigment-forming vibrio..	Human feces	Large curved rod	Slow	Gram-negative	Large, moist, and white, becoming a rich, dark brown	Slowly liquefied	White surface growth, becoming brown and extending to the depths Aerobe and facultative anaerobe	Negative	Slow	.....	Negative

Finkler and Prior, and yet it closely resembles the spirillum of Metchnikovi except that the latter has not a human source.

A PIGMENT-FORMING VIBRIO.

This vibrio is being reported because chromogenic vibrios are rare, if indeed one has as yet been reported. The rectal contents from which it was isolated were obtained by rectal swab from a young male, 18 years old, who arrived January 21, 1912, and who showed some signs of intestinal disturbance.

It is a large, motile vibrio producing a large, white, moist colony on alkalin agar and turning slowly to a dark, rich, brown color from the formation of a pigment. The same grown in alkalin peptone culture medium shows a growth at the surface, which slowly changes to the same dark brown and gradually extends to the depths of the tube. It is an aerobe and a facultative anaerobe, liquefying gelatin slowly. It produces acid in dextrose and saccharose peptone but not in lactose. It is slowly hemolytic and on alkalin blood agar produces a geranium odor similar to that of the growth of *B. pyocyaneus*. It is monotrichous and in this respect resembles the true cholera vibrio. It produces no indol. The only points of resemblance between the true cholera vibrio and this pigment-forming one are (1) the same source, human feces; (2) stain, gram-negative; (3) both are aerobes but the latter is also a facultative anaerobe.

THE DISINFECTANT ACTION OF CERTAIN BACTERIAL STAINS.*

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(From the Bacteriological Laboratory of the Ohio State University.)

HISTORICAL.

Among the earliest workers to note the disinfectant action of bacterial stains were Cornil and Babes,[1] two botanists, who found, in working with methyl violet, that it possessed a toxic action on various bacteria.

In 1890 Stilling published results of work with a dye, probably a mixture of dyes which he called pyoktannin. His experiments, none too carefully performed, with *B. subtilis*, *B. anthracis*, and *M. aureus*, led him to the conclusion that the dye was a general bacterial poison, its antiseptic strength being three times that of bichlorid of mercury, and that the action of the dye was directly proportional to the strength of the dilution used, and to the time of the exposure.

Spina[2] in his article speaks of the use of indigo and methylene blue in the staining of culture media. He uses, however, in 100 c.c. of culture media only a few drops of a sterile saturated solution of the dye, this, of course, being too high a dilution to have any active disinfectant or bactericidal action, even if the stain itself possessed such a property. Spina also notes that in this dilution bacteria have a marked reducing action upon the stain used.

Huber[3] shows the effect of diffused light upon broth cultures of streptococci stained with eosin and erythrosin and exposed to the light for different periods of time as follows: Streptococcus exposed to diffused light for 1 hr. showed growth on first and second day in unstained broth, eosin stained broth, and erythrosin stained broth. Exposure for 3 and 6 hrs. resulted in growth on first and second day in unstained broth, but no growth in eosin stained broth or erythrosin stained broth. The control which was not exposed showed growth in all 3 broths. Results with *B. diphtheriae* were practically the same except that there was only a slight growth in the 3-hr. exposure on the second day.

Conradi and Drigalski[4] deal principally with crystal violet in culture media which inhibited numerous cocci and bacteria, but had no effect upon *B. coli* and *B. typhosus*. A number of organisms belonging to the *subtilis* group are also mentioned among those that grew on their crystal violet plates.

Penzold[5] also notes a definite bactericidal action of certain bacterial stains.

Another work of interest and of more recent date is that of DeWitt.[6] In these experiments with the various dyes, two drops of a 1 per cent solution of each dye are

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† Dr. Andrew M. Jansen died of an infection of glanders on January 4, 1914.

[1] *Mitt.*, Strassburg, 1890.

[2] *Centralbl. f. Bakteriol.*, 1887, 11, p. 71.

[3] *Arch. f. Hyg.*, 1905, 54, p. 53.

[4] *Ztschr. f. Hyg.*, 1902, 5, p. 283.

[5] *Arch. f. exper. Pathol. u. Pharmakol.*, 1890, 26, p. 310.

[6] *Jour. Infect. Dis.*, 1913, 12, p. 68.

used to each tube of agar, and the growth of the various bacteria noted upon the corresponding plate, particular attention being given to the effect of the dye upon culture media.

Much work has been done, especially in the last few months, upon the inhibitive and selective action of certain of the anilin dyes upon certain bacteria in culture media. Churchman[1] shows clearly that the growth of most of the gram-positive organisms such as *M. aureus*, *B. anthracis*, etc., is greatly inhibited when gentian violet is added to the media in the proportion of 1–100,000, while the growth of the majority of gram-negative organisms such as *B. coli*, *B. typhosus*, shows little or no diminution in luxuriance.

In the following experiments, an effort was first made to determine the disinfectant action of the dye itself, without any of the common constituents, alcohol, anilin, etc., which are used when the stain is employed in regular laboratory routine work. The first dye considered was Merck's methylene blue.

Dilutions of the dye were made in sterile water as follows, 1–200, 1–500, 1–1,000, 1–2,000, and 1–10,000. The first organism used was a 24-hour broth culture of *B. coli* recently isolated from feces. Ten cubic centimeters of each of the above dilutions were placed in five sterile test tubes with a sterile pipette. One-half cubic centimeter of the broth culture of *B. coli* was placed in each of the dilutions, shaken up well, and agar plates made from the dilutions at intervals of 3, 5, 10, and 30 mins. In the 1–10,000 dilution the intervals were 5, 15, 30, and 60 mins. each. In making the plate one loopful of the mixture—the dilution to which 0.5 c.c. of broth culture had been added—was transferred to a melted agar tube well shaken, and plate poured as usual. In this manner the amount of stain carried over on the needle would be so small, especially when diluted by the agar content of the tube, that it would have no appreciable inhibitive action.

The plates were incubated at 37° C. for 48 hrs. before examination. The agar used was ordinary meat extract agar, standardized to 1 per cent acid to phenolphthalein. *B. coli* exposed for 3, 5, 10, and 30 mins. to methylene blue in dilutions of 1–200, 1–500, 1–1,000, and 1–2,000 showed growth. In dilution of 1–10,000 *B. coli* showed growth in 5, 15, 30, and 60 mins.

No disinfectant action was manifested by the stain, there being only a slight inhibition in the 1–200 and 1–500 dilutions exposed for 30 mins. The 1–10,000 dilution, exposed for one hour, also showed a slight decrease in the number of colonies on the plate.

In the next experiment, a culture of *M. aureus* recently isolated from a boil was used. The same stain, dilutions, periods of exposure, and technic were used as in the preceding, except that the 24-hour broth culture of the coccus was filtered through sterile filter paper to remove all clumps. The stain had no bactericidal action whatever, the results being about the same as when *B. coli* was used.

Gentian violet was next used in the same dilutions and with the same periods of exposure. *B. coli* and *M. aureus* were used as with methylene blue. *B. coli* was not inhibited appreciably in growth, even in the low dilutions of 1–200 and 1–500, except

[1] *Jour. Exper. Med.*, 1912, 16, p. 221.

in the 10- and 30-minute exposures, and that of 1–1,000 for 30 mins. In the higher dilutions, little inhibition was shown even in the longer exposures.

A marked disinfectant action was shown with the staphylococcus. Inhibition was complete in dilutions up to 1–2,000 at even the shortest exposure, 3 mins. In the 1–10,000 dilution a few colonies were found in the 5- and 15-min. exposures, but none in the 30- and 60-min. exposures, showing that this dilution would have a complete inhibitive action in culture media.

Crystal violet showed about the same selective action as gentian violet, except that *B. coli* showed more colonies in the 30-min. exposure, 1–200 dilution, of crystal violet, than gentian violet; the inhibitive action on *M. aureus* was about the same in each stain.

The effect of dilutions of a sterile aqueous solution of fuchsin was then tried on the same organisms. The lowest dilution used was 1–500, as fuchsin proved to be insoluble in water in the proportion of 1–200.

No disinfectant or selective action is manifested upon either *B. coli* or *M. aureus* with this stain. In the dilutions of 1–500 and 1–1,000 for 30 mins. there seems to be a slight inhibitive action, the 30-min. exposure plate of *B. coli* 1–500 dilution showing no growth.

In the next series of experiments an effort was made to find the disinfectant action of the stains as prepared and used in the laboratory, and containing the usual constituents, such as alcohol, phenol, anilin, etc.

Merck's methylene blue was first used, made up as follows: 30 parts of saturated alcoholic solution of methylene blue and 100 parts of NaOH (1–10,000). In sterile test tubes were placed 10 c.c. of this stain, and 0.5 c.c. of a 24-hour broth culture of each organism used was added to the stain in the test tube, well shaken, and exposed for the following intervals (agar plates were made by taking one loopful from the mixture of the stain and broth culture at the end of each interval): 1, 3, 10, 30, and 60 mins., respectively. Plates were made on ordinary extract agar as before, and incubated at 37° C. for 48 hrs. The organisms used were laboratory stock cultures as follows: *B. coli*, isolated from feces; *M. aureus*, isolated from a boil; *B. typhosus*, isolated in laboratories of the University of Chicago; *B. anthracis; B. mallei*, from the University of Chicago; pneumococcus (Winslow No. 80, American Museum of Natural History). No active disinfectant action was shown by the stain even in the 1-hr. exposures. The pneumococcus and mallei plates contained only a few colonies in the 10-min., 30-min., and 1-hour exposures. The stain as used contained about 22 per cent of alcohol. This dilution, as has been shown by previous experiments, has little or no bactericidal action, and as shown by comparing the last series with the one in which the aqueous solution was used, does not add anything materially to the disinfectant action of the stain.

Anilin gentian violet made up for staining as follows was then used: 1 part of saturated alcoholic solution of gentian violet and 10 parts of anilin water.

Anilin water was made up by using 4 parts of freshly distilled anilin to 100 parts of distilled water. The same organisms, dilutions, and technic were used as in the preceding experiment. Growth was inhibited in each case with anilin gentian violet.

Carbolfuchsin was next used, made up for staining as follows: 1 part of saturated alcoholic solution of fuchsin and 10 parts of 5 per cent phenol.

CARBOLFUCHSIN (as for staining).

Organism	Period of Exposure. 1 min.	3 mins.	10 mins.	30 mins.	1 hr.
B. coli	+++	++	++	+	−
M. aureus	++	++	+	−	−
B. anthracis	+++	+++	++	+	+
B. typhosus	++	+	−	−	−
B. mallei	++	++	+	−	−
Pneumococcus	+	−	−	−	−

+ indicates growth in varying degree; − indicates no growth.

As the aqueous solutions of this stain used in the previous experiments showed little or no bactericidal action, the disinfectant strength of the stain shown in this experiment is undoubtedly due to the percentage of phenol contained. The 1-hour plate of *B. anthracis* showed only a few colonies, probably the result of spores which resisted the action of the disinfectant content of the stain for that period.

In the next work, an effort was made to determine whether some of the ordinary bacteria cultivated in the laboratory are alive and capable of reproduction after they have been subjected to the regular laboratory technic of staining.

Glass cover slips were used as follows: The cover slip was held in a holder which prevented its touching the table or any surrounding object and passed through a flame until completely sterile, and then allowed to cool. After cooling, a loopful of a 24-hour broth culture of the organism was placed on the slip, allowed to dry, and fixed in the usual manner by passing quickly through a flame three times. The slip was then stained in the prescribed manner, depending upon the stain used, the stain washed off with sterile water and the slip dropped into a sterile petri dish with the stained side up. A melted agar tube was then poured in the usual manner, covering the slip. The plates were incubated at 37° C. for 48 hrs. before examination. Control plates were run on slips containing the organism, dried and fixed, but unstained, in order to obtain an idea as to the effect of drying and fixing upon the bacteria.

In the following experiments 24-hr. broth cultures were stained with methylene blue and anilin gentian violet for 1 min., and with carbolfuchsin for 10 secs. Growth occurred in the controls unless otherwise specified. *B. anthracis*, *M. aureus*, and *Pseudomonas pyocyanea* showed growth in methylene blue and carbolfuchsin, but not in anilin gentian violet or Gram stain. In *B. typhosus*, *B. coli*

isolated from feces, *M. melitensis*, and *B. enteritidis*, growth occurred after methylene blue staining but carbolfuchsin, anilin gentian violet, and Gram stain inhibited growth. *B. diphtheriae*, *B. cholerae gallinarum*, *Sarcina tetragena*, and *Microspira comma* showed no growth, but since the control plates with one exception were sterile, the organisms were probably killed by heating and drying.

SUMMARY.

The study of the antiseptic action of the various dyes in the foregoing experiments warrants the following statements:

1. Aqueous solutions of methylene blue or fuchsin have little or no bactericidal action, even in dilutions as low as 1–200 and 1–500.

2. Aqueous solutions of gentian violet have a marked disinfectant action on the gram-positive organisms used in as high dilutions as 1–10,000 exposed for 30 mins. only. In the case of the gram-negative *B. coli* a dilution of 1–500 for 30 mins. was required to show any decided bactericidal action. Crystal violet showed about the same selective and bactericidal action as gentian violet.

3. Methylene blue as made up for staining showed little or no bactericidal action, the percentage of alcohol contained being too low.

4. Anilin gentian violet as made up for staining in the laboratory showed a strong and decided disinfectant action, the 1-min. exposures being sufficient to kill all the organisms used, the gram-negatives as well as the gram-positives.

5. Carbolfuchsin as made up for staining proved also of some value as a bactericidal agent, the action, of course, being almost entirely due to the phenol content.

6. In the cover slip experiments it was rather difficult to determine in some cases whether the disinfectant action was due to the drying and fixing, or to the stain itself. Controls of dried and fixed but unstained organisms were accordingly run. It was found in general that if the organisms in question survived drying and fixing, little effect was seen from the use of methylene blue. Many were killed by carbolfuchsin, and all by anilin gentian violet and Gram stain.

Most pathogenic laboratory organisms which were stained with methylene blue in the ordinary way, if they survived drying and fixing, were alive, capable of reproduction, and would probably not be safe to handle without further sterilization.

All of the pathogenic organisms except spore forms when stained with carbolfuchsin (not dilute) might be handled with safety.

All of the ordinary pathogenic laboratory organisms may be safely handled after being stained with anilin gentian violet for 1 min., or the regular Gram stain.

A STUDY OF TRACHOMA AND ALLIED CONDITIONS IN THE PUBLIC SCHOOL CHILDREN OF NEW YORK CITY.*

BY A GROUP OF WORKERS UNDER THE DIRECTION OF ANNA WESSELS WILLIAMS.

(From the Bureau of Laboratories, Department of Health, New York City.)

(WITH PLATE 2.)

I. Introduction and Critical Review of the Literature.
Anna Wessels Williams.

II. Diagnosis and Course of Four Thousand Cases of Conjunctival Affections Including Trachoma and Allied Conditions.
H. W. Wootton and Anna I. von Sholly, assisted by Caroline R. Gurley, Percy Crane, Alice Kurtz, and Ella Lipsky.

III. Prophylactic Treatment of Trachoma and Allied Conditions in New York City School Children.

1. Special Ophthalmia Classes, School Clinics, Summer Camps.
Anna I. von Sholly, assisted by Caroline R. Gurley, Alice Kurtz, Ella Lipsky, Mary Schmidling, and Theodora Herzig.

2. Personal Hygiene and "Follow-up" Work.
Alice Kurtz, assisted by Ella Lipsky, Mary Schmidling, and Theodora Herzig.

IV. Curative Treatment Including Vaccine Treatment of Infectious Eye Diseases.
H. W. Wootton and Anna I. von Sholly, assisted by Caroline R. Gurley, Olga Povitsky, and Percy Crane.

V. Microscopic and Cultural Studies.
Anna Wessels Williams, Minnie A. Wilson, and Caroline R. Gurley, assisted by Olga Povitsky, Lucy Mishulow, Percy Crane, and Ella Lipsky.

VI. Discussion, Summary, and Conclusions.
Anna Wessels Williams.

I. INTRODUCTION AND CRITICAL REVIEW OF LITERATURE.

Anna Wessels Williams.

Introduction.—Judging from the written word, our knowledge of trachoma is in a most confused state. This is partly due to the fact that, notwithstanding the voluminous bibliography on the subject no minute critical review of the whole has been made for some time, hence the relations between the older observations and the more

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recent ones are not clearly defined. For instance, tho the majority of ophthalmologists consider "Egyptian ophthalmia" a broad term including several different diseases, such as gonorrheal ophthalmia, acute contagious conjunctivitis ("pink eye"), and trachoma, some ophthalmologists still use the term as synonymous with trachoma only. Again, tho the great majority of authorities differentiate between a benign follicular conjunctivitis and a "granular trachoma," some still consider any conjunctivitis with definite follicles as a trachoma, and a few even think that folliculosis, i.e., follicles on an otherwise normal conjunctiva, is "trachoma in stage of invasion."

It is true that Boldt (1904), Axenfeld (1912), and others have given more or less detailed and clear statements of the trachoma situation as recorded in the literature and as gained from their personal experiences, but on the whole they seem to take for granted more definite knowledge than the original records show and hence their deductions appear too positive.

In looking up the literature we have been unable to find reports of consecutive observations on a large series of cases extending over a period of time long enough to include the supposed limits of the course of the longest trachoma cases. The lack of such published records is probably the chief reason why confusion still prevails in regard to several important points. Still a third and of course the most important reason for confusion lies in the fact that the specific cause of trachoma still remains to be definitely proved.

We began our studies about 4 years ago (in the spring of 1910) with these three points chiefly in view: (1) to determine how much we really know of the condition called trachoma; (2) to study the relation of trachoma to allied conditions; and (3) to search for the specific cause of trachoma. Our general plan of work was to study minutely for a long time both from a laboratory and a clinical standpoint a large series of cases embracing "trachoma" and allied conditions. Some of our earlier studies have already been reported, but these papers will give a complete description of the work continued to date.

The critical review of the literature, which is necessarily brief, is taken up under the following heads: (1) Definition, (2) History, (3) Geographical Distribution, (4) Race, Age, (5) Helping Causes, (6) Specific Cause, (7) Symptoms, (8) Treatment, (9) Summary.

1. Definition.

That our knowledge of trachoma lacks exactness is shown at the outset by the different emphasis placed upon the same points in the various attempts at definition. These attempts are made partly from a clinical standpoint, partly from that of histopathology, and partly from a mixed standpoint. For example, Fuchs says (1908): "Trachoma, like acute blenorrhea, is an inflammation of the conjunctiva which originates by infection and produces an infectious purulent secretion. It is distinguished from acute blenorrhea principally by the clinical course in which is developed an hyperplasia of the conjunctiva that forms the most characteristic symptom of trachoma. From the roughness of the conjunctiva caused by this hyperplasia the disease has received its name." In this clinical definition the points emphasized are only two, an infectious purulent secretion and a chronic hyperplasia. Nothing is said about discrete follicles or, definitely, about cicatricial tissue formation. Again, Morax says: "Trachoma is an infection producing nodular lesions (the granulations) situated chiefly on the tarsal conjunctiva, but may be in the conjunctival sacs and even on the cornea. The lesions progress slowly and leave after them very often cicatrices." Here the follicles are chiefly emphasized, and it is admitted that not always is cicatricial tissue formed, and nothing is said about secretion. The Trachoma Commission of Philadelphia agree with Morax, except they state definitely that the process progresses, if unchecked, through its various stages of inflammation to destructive infiltration of the cornea, cicatricial contraction of eyelids and great interference with, if not total destruction of eyesight. Here for the first time pannus is mentioned, and the greatest emphasis is placed upon the destructive changes as a whole. Boldt is more particular and more inclusive. He says: "Trachoma is a chronic deep and dense lymphoid infiltration of the conjunctiva (and tarsus) appearing sometimes diffusely, sometimes as circumscribed masses of cells leading to the destruction of the conjunctiva and its transformation into fibrous tissue, and showing for a time at its onset an abnormal secretion upon which its contagiousness depends." Here follicles are indicated and the hyperplasia leads to destruction of the conjunctiva, that is, to cicatricial tissue formation. Clark, Schereschewsky, MacMullen and others agree essentially with Boldt. Safford sums up the definitions made chiefly from the clinical standpoint thus: "The essential clinical feature of trachoma is a hyperplastic granular destruction of the palpebral conjunctiva."

Axenfeld, giving only descriptions of various phases, does not condense into a definition.

Then come the definitions from the pathologic studies. Thus, Goldsieher says: "Trachoma is a proliferating inflammation of the vascular layer of the conjunctiva in which the perivascular cell infiltration appears in diffuse or granular form." Paschéff says: "The only true trachoma is characterized by a lymphoid tissue richly developed in germinative centers. This tissue is not specific, but is the same as granulations of the pharynx and nasopharynx and experimental follicles." These definitions contradict that of Prowazek who says that the trachomatous process begins in the epithelium of the conjunctiva. Mutermilch tries to correlate these two ideas by the following elaborate definition: "Trachoma consists in a long series of consequent and, in an anatomic and physiologic relationship, unavoidable changes which strive for the establishment of a new and lasting equilibrium between the epithelium and the subepithelial tissue, and which are started by any infectious inflammatory process. It is in no sense different from other chronic pathologic processes in mucous

membranes similar to the conjunctiva. The trachoma process is a complex anatomic change which strives for conjunctival cicatricial tissue."

Mutermilch also calls attention to the obvious fact that the ophthalmologist could never diagnose trachoma in the early stages if he depended upon the definitions given, since most definitions include cicatricial tissue formation.

There is no point on which all are agreed. Therefore trachoma does not stand out clearly as an entity from a study of the definitions. Details, however, give a different aspect to the definitions in several important particulars.

2. History.

Practically all authorities are agreed that early statistics are too indefinite to be trustworthy. Without doubt several quite different diseases were included under one name. Thus Boldt states: "Egyptian ophthalmia included not only the trachoma of the present, but simple catarrh, follicular swelling, follicular conjunctivitis and blenorrhea." From the original memoirs Morax judges that the cases of Egyptian ophthalmia energetically attacking the armies of England, Belgium, Austria, and France in the beginning of the nineteenth century, and considered by many authors as granular trachoma, were for the most part acute contagious conjunctivitis. Fuchs and others make the same statement.

The association of follicular conjunctivitis with trachoma has led to still greater confusion. "Up to 1870," says Morax, "they were confounded." Then arose the dualistic and monistic theories. Later many authors insisted upon differentiating between trachoma, follicular conjunctivitis, and folliculosis, and now practically all ophthalmologists agree on this point. They differ only as to their definitions of the three conditions. Boldt states that the few supporters of the "unitarian" theory (Raehlmann, Mandelstamm, Lawrentzen, and others) who regard follicular conjunctivitis as an attenuated form of trachoma are found almost exclusively in countries where trachoma is widespread, while those who believe in the "dualistic" theory are in countries more or less exempt from trachoma. Until exact definitions are settled upon—whether they be correct or not—we shall still be unable to correlate the reports of different persons.

3. Geographic Distribution.

The same criticisms may be made in regard to statistics on geographic distribution. Until we are at one in our definitions we cannot be sure of our limits of distribution. But the greatest difficulty now is, as Boldt points out, that "owing to the slow and insidious course of many of the cases with a vast number never coming under medical treatment it would require exhaustive investigations of the whole population of a district before reliable statistics could be drawn up." Until this can be done we have to rely upon relative figures and general statements which are often quite misleading.

All countries reported as hotbeds of trachoma (e.g., Egypt, Arabia, Russia, Poland, Finland, Armenia, Ireland, etc.) show also many infections with the Koch-Weeks' bacillus and the gonococcus as well as with many other germs pathogenic for the eye. Hence in these areas it is difficult to determine the specific effects of each infection. Fuchs calls attention to the fact that there are no good statistics concerning the relative frequency of these eye diseases. And we shall not have them, I repeat, until many consecutive cases have been under recorded observation made from minute cultural and clinical study throughout the entire course of each and all of these diseases.

This minute study has been done least of all in those districts where trachoma is reported as endemic. In this country, for example, in the mountains of Kentucky and on the Indian reservations, trachoma is said to be prevalent and increasing, so occasionally one investigator or a group is sent to these areas for a few weeks or months. The result is usually a report of the number of existing cases diagnosed as trachoma, but we are no nearer a knowledge of the real nature of "trachoma" than we were before. If this work could be planned so that comprehensive records started by one group of investigators could be carried on by another over the required number of years we would be in a position to settle the boundaries of at least some of the questions relating to these conjunctival infections, such as insidiousness, intermittence, chronicity, contagiousness, etc.

The Indian reservations are ideal places for keeping such records since they are under direct government control and the individual case can be easily followed. Especially favorable would be the Indian boarding-schools in which Schereschewsky reports such a high percentage of "trachoma," e.g., 90 per cent in one school in Oklahoma.

Of course, until we can show a specific cause we cannot be sure of any statistics for a disease reported to have such manifold variations. But if we have recorded descriptions of the whole course of a large number of consecutive cases we may be able more readily to detach one group of cases from another and to place each group geographically. At present the maps prepared by certain authors are necessarily inaccurate.

4. Race and Age.

In general, authorities are agreed that no race is exempt. According to reports negroes are less susceptible than the other races but this may be because fewer as a whole have been examined. MacMullen says that numbers of well-marked cases in negroes from the West Indies are annually detected at Ellis Island.

All are now agreed that no age is exempt, tho this point also has been disputed.

5. Helping Causes.

There is no dissenting voice from the conclusion that trachoma is a disease above others that is fostered by ignorance and filth. Hence it is found to be first a disease of families among the poor. "The family is the incubator." Cases appearing among the well-to-do are only apparent exceptions. Treacher-Collins cites an instance in point:

"In South Australia, the disease is met with in well-fed, strong, healthy farmers and their families, who reside in roomy, well-ventilated houses. South Australia is, however, a riverless country. Each individual farmer has to arrange receptacles for the storage of surface flood water, and the greatest economy in the use of this conserved water has to be exercised to make it last over the rainless summer months. Where scarcity of water exists, regular and proper ablutions amongst the inhabitants are sure to be neglected and the possibilities of the transference of contagious diseases of the eye are greatly increased. The other accompaniments of a hot, dry district which predispose the conjunctiva to infection, and which aid in the introduction of infective matter into it are also present, viz., frequent dust storms and abundance of flies."[1]

[1] Abridged from Collins' report in the preface of his translation to Boldt's book on *Trachoma*.

According to Morax, "Cleanly Arabs have little trachoma, dirty ones have much." Very recently both MacMullen and Stucky have given detailed accounts of the unhygienic habits of the Kentucky mountains cases, and Schereschewsky, of those of the Indians.

That the disease is of a milder type today ophthalmologists say is due to the more effective carrying out of better hygienic measures everywhere. When we consider how far this still is from the ideal we need not wonder that chronic eye conditions still prevail.

Anything that lowers general vitality, such as tuberculosis, may of course aid any disease. Anything that lowers local vitality appears to be of greater importance in these eye affections. As early as 1861 Weiss considered that trachoma could occur as a sequel of old conjunctival inflammations. Boldt says: "All chronic irritating conditions of the eye, especially obstinate conjunctivitis, augment the tendency to trachoma. . . . . Individual predisposition is increased by external sources of irritation. Among these dust and smoke are of prime importance, for by long-continued action they set up chronic conjunctival irritation. Further, heat, moisture, wind, want of sleep, over-crowding and bad ventilation must be mentioned."

Since unhygienic habits and lowered general and local health also help to produce conjunctivitis from whatever cause, a study of the helping causes of trachoma does not aid in making the disease an entity.

6. Special Cause.

Nearly all authorities agree that trachoma is an infectious disease spread by contact, but a number of investigators doubt that a single specific organism is the cause. The conjunctival sacs are such favorable localities for the growth of several different varieties of microorganisms, especially when invaded by one variety, that the flora obtained from any one examination may be quite large. For this reason it has been easy to admit the theory of multiple causes. Cazalis, who made an extensive study of the etiology of trachoma in 1896, came to the conclusion that bacteria of the various forms of conjunctivitis can, under certain conditions aided by the patient's predisposition, produce trachoma. Later, Mutermilch, Pascheff, and others state that trachoma in no respect differs from other chronic inflammatory processes occurring in mucous membranes whose structure is related to that of the conjunctiva, such as chronic urethritis, atrophic rhinitis, chronic appendicitis, etc. They conclude that trachoma belongs to the class of typical chronic inflammations of the mucosa produced by various causes.

For this reason, too, several other research workers have made claims in favor of one out of a number of known microorganisms as the specific cause of trachoma. Thus the gonococcus, the streptococcus, Koch-Weeks' bacillus, the influenza bacillus (Muller's bacillus), and various other micrococci and bacilli, as well as yeasts, moulds, protozoa, each has had its day, only to be dropped in favor of a new organism when a new investigator happened to find a few cases in which one or the other of these microorganisms seemed to predominate. Some of these hypotheses have lingered longer than others. Some, we have with us still; for instance, the gonococcus hypothesis (Alt. Herzog), tho Morax says (1906): "It is not necessary to discuss the relation of gonorrhea to trachoma. Granulations occurring as the result of gonococcus infection are of short duration and do not cicatrize." Mutermilch on the other hand calls attention to the prevalence of gonococcus infection in the Egyptian trachoma cases

and comes to the conclusion that the gonococcus must have some part in the process. At intervals new claims are brought forward in favor of the gonococcus. The latest, by Herzog, since the discovery of the "trachoma or Prowazek inclusions," is that the gonococcus may become involuted into these granular inclusions.

Claims for ultramicroscopic forms (Raehlmann, Bertarelli) have also been brought forward. Pfeiffer and Kuhnt, however, were unable to get positive results from the use of filtrates into human eyes.

In this report, we are presenting strong evidence in favor of the influence of the group of the hemoglobinophilic bacilli in chronic conjunctivitis—at least evidence that these bacilli may produce a hyperplastic inflammation which may result in cicatricial tissue formation. So we shall give more details of the bibliography of this group as related to the pathology of the conjunctiva.

The Koch-Weeks' bacillus which is placed in this group, tho most authors say it can grow without the presence of hemoglobin (this question of growth will be taken up in the original work), has been frequently reported as occurring in trachomatous eyes, from the time of Koch (1881). Thus Collins (1909) says: "In many cases of trachoma in this country (England) now the Koch-Weeks' bacillus is found in the mucopurulent discharge from the conjunctiva which occurs during an acute exacerbation of symptoms. The ophthalmia introduced by the troops from Egypt was most likely, then, not one disease, but a mixed type." Muller says: "In the majority of adults with trachoma the Koch-Weeks' bacillus was found." Markus states that the Koch-Weeks' bacillus is the cause of "Schwellungskatarrh" (our papillary conjunctivitis). Mutermilch goes further in declaring that "often repeated infection with the same microorganism, e.g., bacillus of Koch-Weeks, produces a series of exacerbations on an already inflamed conjunctiva and finally produces the picture of trachoma."

Many of the bacterial examinations have been made by smears alone, hence the identification of the bacteria was made chiefly from morphology. This means, as we have shown, that many may have been missed. Moreover, successive cultures from the beginning to the end of the disease have seldom been made, neither have hemoglobin-containing media often been employed. Indeed, seldom, if ever, has minute search been made on a large scale either for the hemoglobinophilic group of organisms or for the gonococcus in these chronic eye infections. Earlier reports were taken for granted as representing the whole truth. When we realize that the specific organism may be scanty in the later stages of a disease or that it may be difficult to isolate because more deeply seated or because of over-growth ot secondary organisms we know that we cannot infer much from negative reports based on few examinations. Then, too, and this is a most important point, only a comparatively few investigators have used blood media, and none of them so far as we know, except ourselves, has used an enriching blood medium. Hence, doubtless, we are far from determining the prevalence of hemoglobinophilic bacilli in these eye infections.

Müller, who isolated hemoglobinophilic bacilli from the largest series of trachoma cases reported, recognized the importance of more than one examination. He considered his comparatively small percentage of positive cultures due chiefly to the fact that he could make *only one examination* in many of his cases. He had positive results chiefly in his "acute trachoma" cases. Müller thought at first that his bacillus was the cause of trachoma, but now, without positive proof, most authors have decided that it has nothing specifically to do with trachoma (Zur Nedden, Morax, and

others). Thus Morax says: "It is not difficult to show that this bacillus has nothing to do with the production of granulations." But did he or anyone else show the effect of repeated infections with it on a case of simple follicular conjunctivitis, or on an otherwise susceptible conjunctiva, in producing hyperplasia and finally fibrous tissue? That it is thought to be identical with the influenza bacillus capable of invading secondarily a trachomatous conjunctiva and producing a superimposed influenzal conjunctivitis does not clear up its relation to "trachoma."

In fact, practically no detailed account has been made in the published reports of the number and minute character of attacks of "intercurrent" diseases throughout the course of trachoma and of their relation to the "exacerbations of trachoma." Only general statements have been made of numerous successive attacks of conjunctivitis, both leading up to trachoma and occurring throughout its course. Thus Greef says that the chief cause of conjunctivitis in Egypt is not trachoma but acute catarrh produced by the Koch-Weeks' bacillus and chronic catarrh produced by the Morax-Axenfeld bacillus. Dwyer and Meyerhof point out the difficulty of securing fresh cases of trachoma in any but infants who are apt to show at the same time both gonococcus and Koch-Weeks' bacillus infection. Tho Axenfeld and others recognize the fact that the Koch-Weeks' bacillus and the gonococcus occur frequently in eyes in Egypt and elsewhere, producing acute attacks of "trachoma," they do not show in how many of these eyes these bacilli continue to exist and to produce a subacute or chronic inflammation. Neither do they consider the result of a reinfection with new strains of either of these organisms in eyes already made sensitive to them. All they say is that they *think* that the present "mild type of trachoma" is due to the less frequent occurrence of the Koch-Weeks' bacillus and the gonococcus.

Blumberg in adopting the theory of multiple causes states that recurrent attacks of conjunctivitis diminish the resistance of the reticular connective tissue of the conjunctiva and cause a continual state of irritation which finally produces trachomatous changes. Boldt in quoting Blumberg goes on to say: "If we consider predisposition to consist in overgrowth of adenoid tissue, as Peters does, Blumberg's theory becomes very plausible, and we can readily understand how repeated chronic inflammatory conditions of the eye produce sponginess, atony, and a tendency to proliferation in the adenoid tissue."

We can clearly see from the above brief sketch that the question as to the ultimate influence of these bacteria on the eye is far from settled. We have demonstrated the continued presence of hemoglobinophilic bacilli in cases showing successively acute, subacute, and chronic inflammation of the conjunctiva and their increase in numbers during acute exacerbations. The following reports take up this question more fully.

In the meantime came the work of the *inclusionists*. The bibliography of this work has been gone over so fully both by this laboratory and by other sources that the chief points may be only touched upon here.

Halberstaedter and Prowazek (1907) described certain bodies in the conjunctival epithelial cells of cases diagnosed by them as trachoma. Greef, Claussen, and Frosch independently described similar bodies. Unfortunately they did not go into details as to the course of their cases so we do not know just where to classify these in a disease of such protean descriptions as trachoma. Halberstaedter and Prowazek simply state that the inclusions were found more frequently in fresh cases. The same indefiniteness as to clinical course is shown by most of the authors following them.

We can only gather that the bodies were found in largest numbers in "acute trachoma" or in "acute exacerbations of old trachoma."

Then came others who said that similar inclusions are found in acute conjunctivitis cases of the new-born, also in vaginitis, urethritis, and even in the normal conjunctiva.

As is usual after the report of a new finding confusion has arisen as to how many manifestations may be considered a part of the described bodies.

From the accumulation of evidence gained from the vast amount of work done by others on this subject as well as from our own work extending over three years we have come to the conclusion that there are inclusions in the conjunctival epithelial cells of certain cases of conjunctivitis, answering in the main to the descriptions of Halberstaedter and Prowazek and others, which are foreign invaders. We further believe that these foreign invaders may enter the epithelial cells of other mucous membranes of the body but that they could not enter the normal cell in appreciable numbers without producing an inflammatory reaction. Hence we believe that the bodies found in the normal conjunctiva (McKee, Addario) are simply incidental cell changes. Indeed the descriptions and plates of the above named authors show this.

We have already called attention to the similarity of the "typical inclusions" found in our cases of papillary conjunctivitis ("acute trachoma") to nests of the hemoglobinophilic bacilli. This work will be taken up more fully in the body of the report. This idea differs from that of Prowazek in regard to their nature. He has created for them a group which he calls the Clamydozoa—or mantle animals—and places it between the protozoa and the bacteria. Prowazek seems not to be aware of the limits of morphologic changes in bacteria.

Apparently we have no definite help in making trachoma an entity from the studies of its etiology.

Nor does the study of the reports on the *contagiousness* of trachoma help us. While all but a very few authors agree that trachoma is contagious they differ as to the degree and stage of contagion. Only general statements can be considered since the few specific statements are not backed up by enough clinical evidence to draw trustworthy conclusions. Thus Boldt says: "Experience teaches that infection does not readily occur but usually only after prolonged contact with the patient and common use of beds and washing utensils. Then one person after another is slowly infected, therefore we can only claim a restricted and conditional contagiousness for trachoma, not underestimating the significance of individual predisposition." So too, Axenfeld, Morax, Fuchs, Doyne, Clark, and most others believe that trachoma must be placed among the less contagious, or slowly contagious diseases. On the other hand a few authors say that it is highly contagious, e.g., Trachoma Commission of Philadelphia, etc.

All agree that "the danger of contagion is in direct proportion to the amount of secretion," for "the specific secretion may be enhanced by a superimposed infection." But the possibility that the "superimposed infection" may produce a hyperplastic or an atrophic inflammation seems to be lost sight of except by the few. It is true that Morax comes near it when he calls attention to the light cases—the subacute and chronic cases of Koch-Weeks' bacillus infection which "are considered non-important but which may give rise to more serious ones."

In these subacute and chronic cases, as we have shown, the secretion may be so slight that it may be easily overlooked, and even when found, the stained spreads from

it may show no recognizable bacteria. Cultures, however, may show that bacteria are present. These bacilli with their possible change in virulence may insidiously attack another eye which may be in a condition to allow a chronic inflammation instead of an acute one.

We know too little either about the condition producing virulence in certain groups of bacteria, or about the conditions influencing susceptibility to their pathogenic action summarily to exclude them from a list of causes producing any degree of proliferative and fibrous changes in tissues.

Symptoms.

Once more most statements are naïvely limited or indefinite. The disease is divided into types, severe and mild, papillary and granular, acute and chronic, etc., and these types into stages such as development, regression, cicatrization, etc. Different terms are used for apparently the same type and stage by different authors, which makes the confusion worse. The trouble is that the course claimed for this disease is so protean in its variations and as Collins points out, is "usually of such exceedingly long duration, that few observers have had the opportunity of watching cases from commencement to finish," and therefore of getting at a clinical entity if it exists. These confused ideas may be more easily seen by a consideration of the claimed symptoms.

Onset.—Some authors say the disease probably always begins acutely with secreting conjunctivitis (Collins, Reese, Schereschewsky, etc.). Others are just as emphatic in stating that it always begins insidiously with follicle formation and without visible secretion (Weeks, Trachoma Commission of Philadelphia, Stiel, MacMullen, etc.); while a small majority take the middle course and consider that tho there undoubtedly are acute secreting cases they are now few (Boldt, Morax, Fuchs, May, Harrison, etc.). But since all authors are agreed that "one rarely sees a beginning follicular or granular trachoma" and follows it to its terminal cicatrization, it can be seen that the character of onset as a whole in this disease must still remain a question.

Secretion.—While there is much doubt as to how often, if ever, trachoma begins with a secreting conjunctivitis, there is very little difference of opinion as to the presence of a "specific secretion" from time to time during the course. But how to differentiate between a "specific secretion" and that caused by a superimposed infection seems difficult. In fact, few attempt it and these most unsatisfactorily. Thus Morax says: "At a certain degree [?] of development there is always produced a slight mucopurulent secretion. When the secretion is more abundant [?], when the lids stick much [?] on waking, it is nearly always the result of superimposed infection. . . . . Probably all cases characterized by a purulent or mucopurulent secretion are mixed infections." Of course the majority of ophthalmologists say that they depend upon the microscope for differential diagnosis but this generally means one examination by smears alone, and as we have shown above, this is insufficient to rule out a number of bacteria. Stiel states: "In pure trachoma secretion of the conjunctiva is not necessary; on the contrary, secretion always means a mixed infection." Considering the prevalence of bacteria causing all grades of secreting conjunctivitis, the reported susceptibility of trachomatous eyes to mixed infections, and the prevalence of all conditions favoring such infections, it is highly improbable that anyone could demonstrate the course of any case of "pure trachoma" going on to cicatrization without secretion appearing at times throughout its course. The

statements, therefore, "that secretion is not necessary" and "that only a slight secretion is specific" seem to be matters only of opinion.

Follicles.—An immense amount of study has been carried on concerning the characteristics of follicles in trachoma with the result that no definite clinical or microscopic differences between the follicles of "trachoma" and of other conditions have been demonstrated. While the majority of authors believe that there is essentially only one form of trachoma, that is, "granular trachoma," they cannot agree in differentiating trachomatous granulations from follicles found in any follicular conjunctivitis except by stating that the former are "follicles which lead to permanent injury of the eye," which opinion is of no assistance in early diagnosis.

Cicatricial tissue formation.—Here we have a difference of opinion again. Some authors state: "Always some cicatricial tissue is formed, however light the case and whatever treatment" (MacMullen, MacCallan, etc.). Others think with Boldt that "we no longer consider connective tissue formation and scarring to be an inevitable result as Saemisch once did, because it is frequently possible by early and appropriate treatment to stop the disease before the appearance of the cicatricial stage." Then it has been shown that certain known infections may produce cicatricial tissue, e.g., Morax-Axenfeld bacillus and gonococcus in the eye, influenza bacillus in the lungs, etc.

Pannus.—Here, tho there may be a question as to the limits of "specific pannus," practically all are agreed (even those who put pannus under complications and sequellae [*sic*]) that such a pannus exists but that it seems to have no relationship to the intensity of the infection as evidenced by follicle formation since it may occur at any time throughout the course; however, it is supposed to be more serious when it appears late in the disease, i.e., after the establishment of cicatricial tissue.

Diagnosis.—Considering all of the above conflicting statements in regard to symptoms, there is little wonder that when it comes to a question of early diagnosis, no one seems to be able to give any aid. How many of these early cases are followed up to determine whether, according to any of the various opinions, the diagnostician is right or wrong? Consequently, all authorities are agreed that "in the beginning the diagnosis must be held in suspense." "On one examination a certain diagnosis can only be made in old typical cases," that is, in cases having cicatricial tissue or "typical pannus" or both. This is simply saying that eyes showing such changes are trachomatous and leaving out of consideration the question as to how many different agents may cause pannus and conjunctival cicatricial tissue.

Prognoses.—These, of course, are various. Some say there may be spontaneous cures; some even claim that there are abortive cases which heal without scars; others assert that unless treatment be instituted in a very early stage trachoma is never cured; some say that with early treatment there may be complete recovery without scarring; others say that however early the treatment, there is always scarring, etc. Again we may take our choice.

Treatment.

Here we have another rather wide limit of choice. As far as prophylactic treatment is concerned all are agreed as to its importance, but few have carried it into full practice. Most of the little that has been done has been directed against follicles and not against acute conjunctivitis or the lighter subacute cases where no follicles may be present in the beginning. Until we so realize the importance of the relationship of acute conjunctivitis to trachoma that we shall employ aids on every side such

as are shown in our reports on prophylaxis (see p. 281) we may expect tardy results, in ridding us of "trachoma."

The great majority of the treatises on treatment have to do with more or less advanced cases—with curative treatment. Trachoma is said by most authorities to be comparatively easily cured leaving cicatricial scars after at least several months of treatment under the following conditions, (1) when there are no complications, (2) when in children, or (3) when cases come early enough. All of which statements might be applied to any form of chronic conjunctivitis.

The principle of treatment in the advanced cases is that used in any chronic sluggish inflammation—stimulation. Several authors emphasize the importance of change of stimulants. On the other hand Boldt says that early slight cases or chronic torpid ones often do better under simple hygienic treatment.

Opinions differ now so widely in regard to the merits and applicability of operative treatment, i.e., expression, grattage, excision, that a study of them gives us little help in determining the entity of trachoma.

Summary.

In summing up the mass of writing on trachoma we find that there is no widespread agreement as to the claimed essential points. The chief lack seems to be that there has been no critical following up of a large series of cases over a long period of time with studies directed especially to the following particulars:

1. Written records of each case from the beginning to the end of the disease.

2. Successive cultures as well as microscopic examinations made of each case using enriching media to detect those organisms that grow with difficulty, combined with inhibiting substances for organisms which might overgrow more important ones.

II. DIAGNOSIS AND COURSE OF FOUR THOUSAND CASES OF CONJUNCTIVAL AFFECTIONS INCLUDING TRACHOMA AND ALLIED CONDITIONS.

H. W. Wootton and Anna I. von Sholly

assisted by

Caroline R. Gurley, Percy Crane, Alice Kurtz and Ella Lipsky.

To the medical profession it seems hardly necessary to state that the diagnosis of trachoma, except in the cicatricial form, is an exceedingly difficult matter, the more so in view of the fact that the existence of such a pathological entity has recently been doubted. Is there or is there not a specific chronic inflammatory affection of the conjunctiva frequently accompanied by a characteristic involvement of the cornea, which begins with thickening of the adenoid layer and the localized accumulation of small round cells of various kinds, which are gradually transformed into granu-

lations and cicatricial tissue? This question has been answered in the affirmative by most of the better known writers upon ophthalmology, and this description of the disease has been regarded as a classic. Recent investigations, while not conclusive, have tended to throw doubts upon this time-honored theory and to suggest that trachoma, far from being a disease *sui generis*, consists rather of a series of infections possibly of similar, perhaps of dissimilar character, from which, at certain stages, patients may recover spontaneously, or be cured by treatment, but which, after a certain period, result in cicatricial formation. This theory tends to explain why, altho trachoma is on all sides believed to be contagious, no specific organism of the disease has been discovered despite the most painstaking search. It also coincides with the known fact that trachoma is a disease of no special race or country but is essentially a manifestation of uncleanliness and insanitary surroundings. A person of ordinarily cleanly habits who contracts, let us say, an acute catarrhal conjunctivitis, and pays proper regard to cleanliness and treatment, is speedily cured and is not liable to immediate reinfection from his surroundings. The case is different, however, with the poor living in hovels in Eastern Prussia. Here, an attack of acute catarrhal conjunctivitis, not receiving proper treatment, would have a tendency to pass into a subacute or chronic stage, would infect other members of the family, and would, in turn, be reinfected by them. Thus the irritation caused by the original infection would tend to become more or less continuous and, unless checked by treatment and a change in hygienic and sanitary conditions, would ultimately result in the production of cicatricial tissue. This theory is as difficult to combat as it is to prove for the reason that, until someone has been able to follow closely a sufficient number of cases of conjunctival affections from their incipiency to their termination in cicatrization, and at the same time to rule out other infections which might produce fibrous tissue—a study which, so far as we know, has never been made—we shall not be able to state that trachoma as a pathological entity exists.

The studies reported in this series of papers give the results of continued observation of a large number of cases.

At the beginning of this work we established a special clinic in connection with the Research Laboratory in order to allow clinical and laboratory observation to proceed closely together.

Here we developed the folder system of records, very valuable in this kind of a study. The outer folder (see p. 275) contains the previous history of the case, the family history, notes of home visits, and the original and supplementary diagnoses of the case with the results of microscopic and cultural studies; while the inner cards, which may be added as needed, contain a minute description of the different phases in the course of the case.[1]

Even with these descriptions, it has been at times difficult for us to come to an understanding as to each one's meaning of hypertrophy, thickening, cicatricial tissue, characteristic follicles, kinds and amount of secretion, etc. For example, is the term hypertrophy used to mean true hyperplasia, that is, proliferative inflammation with the formation of cellular connective tissue, or may it also be used to mean only a more or less temporary infiltration of lymph cells? Clinically, of course, it may be impossible to decide this question until late in the course. Again, we found that a small amount of secretion gathered in the superior *cul de sac* was not recorded by some examiners; the fact of its presence is of importance in accounting for the finding of hemoglobinophilic bacilli in cultures from those chronic cases reported as having no visible secretion, and in accounting, too, for a focus of infection.

In this way more or less accurate and continuous observations, extending over a period of about 4 years, have been made of some 4,000 cases of conjunctival affections occurring for the most part in the public-school children of New York City. These observations tend to confirm the statement of Fuchs and others that in children trachoma as it is described in its entirety is a rare disease.

The affections of the conjunctiva most common in this series of cases which have, from time to time, been regarded as trachoma by various authors, may be described under five headings.

[1] During the first half of our work all of the diagnoses and descriptions were made by the one in charge of this part of the work (Wootton), but latterly other ophthalmologists have assisted, and finally when the ophthalmia school and the school clinics were started this part of the work was definitely in charge of another of the group (von Sholly). Only doubtful cases have been referred to the director who visited the clinics from time to time. It can be readily seen how essential are full written descriptions of each case.

OUTER FOLDER (FRONT)

Name	Place of Birth	Age	Public School	Date Admission	Case Number
Celia K.					
Address	*U.S*	*9 Yrs.*	*22*	*5/27/11*	*1436*

X

DIAGNOSIS

1. Original (*a*) Clinical *5/27/11 Follicular Conjunctivitis (mild)*
   (*b*) Microscopic + "*trachoma inclusions*," + *hemoglobinophilic bacilli*
2. Complications ——
3. Supplemental Diagnosis *7/27/11 Papillary "trachoma" (later called papillary conjunctivitis)*

HISTORY

4. When did eye trouble begin? *Dec. 1910*
5. Subjective Symptoms (*a*) Onset *Ocular injection; lids stuck in mornings*
   (*b*) Now
6. TREATMENT (*a*) When? *Dec. 1910* (*b*) Where? *Gouverneur Clinic* (*c*) Nature of Treatment
7. OPERATIONS (*a*) How many? —— (*b*) Where Performed? —— (*c*) When? ——
8. DISCHARGED—(*a*) Date *1/28/13* (*b*) Condition *Normal*

10. RELATIVES

X	Relationship	Name	Age	Place of Birth	Employment or School	Time in U. S.	Date of Exam.	Eye Condition	Case Number
	Father			*Austria*		*12 Yrs.*			
	Mother			"		20 "	*4/2/11*	*O.K. 6/7/11 O.K.*	
	Sisters	*Sadie*	*10*	*U.S.*	*P.S. 98*				*1165*
		Rose	*8*	"	" "		*4/1/11*	*O.K. 6/5/11 O.K. 7/26/11 O.K. 10/5/11 O.K.*	
		Lillie	*6*	"	" *88*		"	*O.K. 6/5/11 O.K. 7/26/11 O.K. 3/28/12 O.K.*	
		Esther	*15 mos.*	"			"	*O.K. 6/7/11 O.K. 7/26/11 O.K.*	
	Brother	*Peter*	*3*				"	*O.K. 6/7/11 O.K.*	
	Gr'dfather						*6/7/11*	*O.K.*	

11. No. of Boarders *0* (*a*) Nationality (*b*) How long in U.S.?
12. HYGIENIC SURROUNDINGS (*a*) No. of Rooms *3* (*b*) Other Occupants of Sleeping Room Adult *0* Children *1* (*c*) Does Patient Sleep in Separate Bed? *No*
(*d*) If not, with whom? Child 1 Adult (*e*) Separate Towels? *No* (*f*) Separate Pillow Cases? *No* (*g*) CLEANLINESS *No*

OUTER FOLDER (BACK)

INSTRUCTIONS

When further comment on point is desired put "X" in column marked "X" against the point, and enter number in column marked "X" on Supplementary page.

Write first—date of interview, name of person interviewed (giving initials) and address.

Paragraph freely.

Do Not Write in This Space	X	SUPPLEMENTARY NOTES
Home Visits		
4/1/11		*See front of sheet.*
5/27/11		*December last, eyes red and stuck in mornings; went with sister to clinic; not told nature of trouble; mother says no trouble now; never was kept out of school; mother says redness lasted only a day or so.*
6/5/11		Celia. *Tarsal conjunct. practically normal except for few fine folls.; lymphatic dilatations lower lids.*
		Lillie *O.K.*
6/7/11		Sadie. *Tarsal conjunct. normal color, vessels seen, moderate number translucent folls. on tarsus and in canthi. Lymphatic dilatations lower lids. Follic. Conj.*
7/26/11		Celia. *Conjunct. upper lids very much thickened; papillary condition; vessels not seen; mucopurulent secr.; lymph. dilatations of lowers. Looks like "Trachoma." (Goes to Gouverneur Clinic three times a week.)*
		Sadie. *Tarsal conjunct. somewhat thickened, with numerous folls.; vessels obscured; slight secretion; ptosis right eye.*
		Mother very shiftless and indifferent.
9/25/11		Celia. *At Convalescent Home at Wilton, Conn.*
10/10/11		" " " " " " " *; has gained 4 lbs.*
2/24/12		Celia. *Eyes still paste in morning.*
3/28/12		*Mother preparing for holidays. Walls just painted, but everything else extremely untidy and dirty. Mother seems rather suspicious or indifferent, certainly not interested in regard to instructions; says too busy to attend demonstrations now. Eyes O.K. (but condition favorable for re-infection).*
		Celia *and* Sadie *now go regularly to housekeeping classes at 226 Henry Street.*

FOLLICULOSIS.

This is a pronounced type of follicular formation without evidences of inflammation. The writers would describe follicles clinically in this group of cases as situated either upon the surface of the conjunctiva or in its substance. They may be situated on any part of the conjunctiva of both lids but everywhere arise from a normal base. The underlying conjunctiva is not thickened and its blood vessels are perfectly distinct. In the upper, and more especially in the lower, fold of transition, hemispherical or ellipsoidal, yellowish translucent bodies may also be discernible. These are less firm than the follicles and are simply an accompaniment of the follicular affection and may be or may not be present. They are also sometimes observed upon the ocular conjunctiva, and are said to be simply dilated lymphatics. There is no secretion and the eyes are not injected. There are no clinical symptoms even when follicles are present in a marked degree. This form of conjunctival affection in itself never terminates in cicatrization and is never accompanied by corneal involvement. That these cases, uncomplicated, are harmless is shown plainly by the course of our series of cases, given in Table 1. Only three of these cases were operated upon, and only two showed, about one year after first seen, a conjunctivitis during which pannus developed. All of the others—over 2,000 cases—have now either normal conjunctivas, or only a few small scattered follicles.

FOLLICULAR CONJUNCTIVITIS.

In this variety, follicles are present as before and the "lymphatic dilatations" may or may not coexist. The mucosa, however, is injected, secretion, for the most part watery, is present, and the eyes appear suffused. Mucous or mucopurulent secretion may also be present from time to time. There is no thickening of the conjunctiva surrounding or underlying the follicles. These cases would, according to the theory above described which denies the existence of trachoma *per se*, be capable, unless cut short by treatment, of becoming hyperplastic and terminating in cicatrization. One occasionally, tho rarely, sees in children cases presenting at the same time the follicular formations, secretion, and pannus.

TABLE 1.

Chief Clinical Points in Course of 4,261 Cases under Observation.

Original Diagnoses	Later Diagnoses	Number	Follicles	Pannus	Cicatricial Tissue	Operations		Present Condition				Number Lost Sight Of
						Before Seen	After Seen	Unimproved	Improved	Cured		
										Number	Average Time	
Old trachoma	Old trachoma	59	48	32	59	25*	3	10	36	4	3 yrs.	9
Acute, hypertrophic, or papillary trachoma	Papillary conjunctivitis	181	162	8	58†	64	6	8	19	136	9 mos.	18
Doubtful trachoma	Papillary conjunctivitis	161	161	3	49†	58	9	6	15	123	10 mos.	17
	Follicular conjunctivitis	20	20	0	5†	7	0	0	0	19	1 yr.	1
Follicular conjunctivitis	Papillary conjunctivitis	16	16	1	3†	4	2	2	6	7	1 yr.	1
	Follicular conjunctivitis	665	665	0	45†	51	3	0	69	596‡	1 yr.	(?)‡
Acute catarrhal conjunctivitis	Papillary conjunctivitis	20	8	2	2†	3	1	1	4	15	6 mos.	0
	Acute catarrhal conjunctivitis	700	105§	0	0	0	0	0	0	700‡	6 wks.	(?)‡
Folliculosis	Papillary conjunctivitis	5	5	2	2†	3	0	0	2	3	6 mos.	0
	Folliculosis	2,120	2,120	0	0	0	0	0	0	2,120¶	1 yr.¶	(?)‡
Miscellaneous	Blepharitis, phlyctenular conjunctivitis, etc.	300	190	0	0	0	0	0	0	294	3 mos.	(?)‡

* It was often impossible to get clear histories from these old cases. Probably all have had at least one operation for expression.

† Only operative scars presumably.

‡ These cases were not all examined at the present time. The great majority, at least, are still in our schools and are sent to our clinics from time to time for examination, and immediately, if inflammatory symptoms supervene.

§ Mild folliculosis, chiefly, after acute process had subsided.

¶ With the exception of mild folliculosis in a comparatively small number of cases.

This fact, according to the point of view, may be held to support either the theory in question or the theory that there is such a pathological entity as trachoma.

In most of those cases which, according to our observations, have presented conjunctival thickening (hyperplasia?) as well as follicular formation, the follicles have been larger than in the cases we are prone to call follicular conjunctivitis. In some adult cases which one of us (Wootton) has seen, the presence of follicular formation deep in the conjunctiva itself has been evidenced by the occurrence here and there of small yellowish infiltrates. Such cases the observer called trachoma, since pannus had been seen to occur in several of them. No case clearly of this type has been seen in the series of school children here studied. Out of the 671 cases diagnosed when first seen as follicular conjunctivitis, 16 developed later a subacute papillary condition all of which, except one, cleared up without showing pannus.

PAPILLARY CONJUNCTIVITIS.

The characteristic feature is thickening of the conjunctiva, presumably through infiltration with lymph cells and wandering connective tissue cells. Follicles may or may not be visible and when present do not form an essential feature of the disease. The conjunctiva is thickened and red; its blood vessels are invisible or obscured; mucopurulent secretion in varying degree is always present tho sometimes in so small an amount that only an occasional flake can be seen in the superior *cul de sac*. In these cases the so-called trachoma inclusions or bodies of Halberstaedter and Prowazek are so frequently present that they may be regarded as a constant feature. For this reason, in the early part of the studies upon which this article is founded, these cases were regarded as trachoma.

In following the course of these cases, we found that practically all of them recovered after presenting a subacute or chronic condition for a varying number of months, *without having developed either pannus or cicatrization*. All of these cases were treated, most of them from the beginning, both at home and in the clinic in our usual way (p. 281), hence we cannot be sure what course all of

them might have followed without such treatment. All we can say is that our observations lead us to support the suggestion of Cohen and Noguchi that "trachoma inclusions" are microorganisms which may produce a conjunctival affection having nothing to do with classic trachoma. From the work of Williams and others of our group (p. 329), it would seem that the "trachoma inclusions" in this type of cases are nests of hemoglobinophilic bacilli growing in the epithelial cells of the conjunctiva. This latter hypothesis supports the observations of Markus and others who have stated that members of the group of hemoglobinophilic bacilli are the cause of papillary conjunctivitis (*Schwellungskatarrh*).

TRACHOMA.

These are the cases which present the well-known characteristics of cicatricial degeneration or corneal involvement or both. In our table we have classed all of those that showed these characteristics when first examined by us as "old trachoma," excepting 8 cases of papillary conjunctivitis with pannus which gave a history of moderately short course. The few cases of cicatricial tissue formation which appeared during our 4-year period of observation were post-operative cases and the scar tissue apparently was a result of operation. These and the pannus cases are indicated in the "course" columns of Table 1. From the study of the group of cases classed as "old trachoma" in our table, we can infer little, since we were not able to study them from the beginning. But from those proceeding to cicatricial tissue formation (without operation) and pannus while under our observation we may hope to gain more light.

In favor of the classical theory that trachoma is a disease *per se*, we must not lose sight of the peculiar character of the corneal involvement which so often occurs when the conjunctiva shows cicatricial changes. This keratitis, designated by the special name "pannus," is described as an infiltration of small cells between the superficial epithelium and Bowman's membrane, which proceeds from above and is accompanied by vascularization from the adjacent conjunctiva. It may gradually invade and destroy Bowman's membrane. In our series of cases we have found a

condition answering to this description in 48 cases. In 16 there is an acute history and as yet no cicatricial formation has been observed in any of them. Eight of these 16 cases developed the pannus while under our observation, and the other eight showed beginning pannus on first visit and gave a history of a moderately acute process. The other 32 cases were all old cases when first seen by us. The microscopic findings in these cases are given on p. 315.

Let us now turn for a moment to the consideration of the corresponding conjunctival affections of adults. The pure follicular type (folliculosis) is exceedingly rare, but when it does occur is no more liable to disagreeable sequelae than in children. The second type, follicular conjunctivitis in a pronounced form without thickening of the conjunctiva, is, we should say, practically unknown in persons over 16 years of age. Papillary conjunctivitis is found in adults and presents the same characteristics as in children.

The terminal stage of trachoma, represented by cicatrization of the conjunctiva and pannus, is much more common in adults than in children. Most of the cases in New York City occur among the foreign population probably as the result of infection in foreign lands. These cases are decidedly less frequent in our clinics at present than they were 10 years ago, and there can be no question that this desirable state of affairs has been in part due to the strict enforcement by the immigration authorities of regulations prohibiting the entrance of trachoma into this country, and in part due to the stricter carrying out of rules of personal hygiene in schools and homes, and the greater care of eyes in the clinics. The terminal stage of trachoma is, however, still seen in adults who have lived in the city all their lives and who, so far as can be judged from the history, have contracted the disease during adult life. We naturally ask ourselves what characteristics these cases presented in their incipiency and previous to the stage of cicatrization. The immigration authorities, judging from such of their cases examined by us, regard thickening of the conjunctiva, especially that of the retrotarsal fold, as the most characteristic symptom of incipient trachoma. The detection of slight thickening in this location, however, is sometimes a difficult question and a

matter of personal opinion, and for this reason doubtful cases are isolated and observed for a period of some weeks before the final diagnosis is made.

In our series of cases a number that have shown clinically deep-seated follicles and a thickened retrotarsal fold have apparently recovered without a trace of cicatricial tissue in evidence.

SUMMARY.

1. Several processes in the conjunctiva of children, evidenced clinically by a more or less chronic thickening or follicle formation or both and formerly considered as a part of a chronic process called trachoma, may run a comparatively quick course to cure without the formation of cicatricial tissue.

2. Trachoma as it is described in its entirety is very infrequent at present in the public-school children of New York City.

III. PROPHYLACTIC TREATMENT OF TRACHOMA AND ALLIED CONDITIONS IN THE NEW YORK CITY SCHOOL CHILDREN.

1. SPECIAL OPHTHALMIA CLASSES, SCHOOL CLINICS, SUMMER CAMPS.

Anna I. von Sholly

ASSISTED BY

Caroline R. Gurley, Alice Kurtz, Ella Lipsky, Mary Schmidling, and Theodora Herzig.

SPECIAL OPHTHALMIA CLASSES.

Practical measures on a large scale for the prevention of trachoma and other infectious eye diseases date from the beginning of the nineteenth century. They were first promulgated by the military authorities as rules of hygiene for the army in order to keep in control the more or less severe epidemics of ophthalmias that played havoc with the troops. Since soldiers are recruited from and discharged back into the civil population, the next logical step was to extend the supervision of the state to the latter.

With the increased knowledge of etiology and prophylaxis of infectious diseases, measures more or less efficient are being taken

in all advanced countries to ward off epidemics, not only of trachoma, but of all infections in public and in charitable institutions where numbers of people are collected together. Thus, in the twentieth century all nations that stand at the front have certain regulations for the inspection and care of their public-school children. It is obvious that through the army and the public schools the state and municipality have the easiest and most direct control over a large proportion of the population. Each unit is in more or less close relationship with several units of the rest of the population through the family and neighbors.

The details of the method of control for the suppression and extermination of eye infections in the various countries, as well as their immigration regulations, are fairly well known and can be obtained from the attached bibliography. It is enough to say that as the result of the measures used trachoma is seen only sporadically in urban centers and not frequently seen in the country districts except in certain foci mentioned by Dr. Williams where the economic and hygienic conditions are of a very low grade. Large epidemics, such as were known before the early nineteenth century, do not occur. Small epidemics occur in an occasional institution or town, but are quickly curbed. However, so long as these foci exist and migration continues to grow easier we cannot afford to relax our guard.

Dr. Williams has given the various theories of the etiology of trachoma. Whichever be correct, it is agreed that contagion is carried through the medium of the conjunctival secretion. Moreover, the logical deduction from our theory as formulated by Dr. Williams makes it incumbent on us to give much more consideration than hitherto to our methods of handling the acute and subacute conjunctival inflammations.

The general rules governing the prophylaxis of infectious eye diseases are those of segregation of the affected during the secreting stage and the enforcement of personal and domestic hygiene. Recommendations for bodily cleanliness, especially of hands and fingers, clean personal habits, individual bathing utensils (sponges, washcloths, towels), the use of individual bed clothes, pillowslips, sheets, etc., are found in every book and article written on the

prevention of these diseases and some of our methods of enforcing these recommendations are given in the succeeding paper.

The particular contribution which we have made in the prophylaxis of trachoma is in the establishment of special day classes in the public schools of New York City for children suffering from any form of conjunctival infection. Thus these children may be kept under control, segregated, treated, and taught prophylactic hygiene.

The method of handling infectious eye diseases in all the cities of this country and Europe, where attention is paid to the matter at all, is to exclude all children showing conjunctival secretion and simply to advise treatment. A certain number of cities have an inspecting nurse or doctor teach the child and its family the methods of transmitting infection. When the actively secreting stages pass, the child is readmitted to school and if not completely cured is told to continue under treatment. Of course this means that a number of subacute and chronic cases with secretion too small in amount to be easily detected may continue to be a menace to others.

In 1870 in England, Mr. George Critchett, finding a large number of eye infections in one of the schools, advised the establishment of "a ward or separate school" where these cases might be isolated, treated, and still receive their education. In 1873, such a school was tried for a year with most satisfactory results. Not much was done after this on a large and more formal scale until 1888, when an isolation school for 400 children was opened at Hanwell. The surgeon in the school reported a decrease in ophthalmia from 35 per cent in 1889, to 7 per cent in 1893 and 0.5 per cent in 1895. Later, as the result of the success of the school, provision was made to take care of the children of the poor-law schools who were suffering from ophthalmia, and two isolation schools were erected in 1903 and 1904 at Swanley and Brentwood, respectively, where the affected children are still being isolated, treated, and educated. These, however, are resident schools.

In New York City there are more than 700,000 public-school children. The 1912 Health Department report showed that 33,860 of these children had suffered from acute contagious con-

junctivitis during that year, and 15,245 from so-called trachoma ("granular lids," which included folliculosis and follicular conjunctivitis). The larger part of the sufferers live in the poorest and the most congested parts of the city. Their surroundings and habits are such that each one is a focus of contagion to his neighbors.

Our school children have been under periodic and careful examination for eye diseases by the Health Department since 1902 and all children considered a source of danger to others have been excluded from school until certified cured by a physician. As far as her authority has carried her, the school nurse has forced every child diagnosed as suffering from trachoma, follicular conjunctivitis, blepharitis, acute conjunctivitis, keratitis, etc., under medical care either of the family doctor or a public dispensary. To obviate any financial hardship, the Health Department established free eye clinics in the congested districts. The children not excluded but under treatment are obliged to show the nurse at intervals a signed note from a doctor or a dispensary card stamped with date of treatment to prove that they were under medical care. The excluded children have been supposed to report daily in the school nurse's office to show the progress of their case. As far as possible home visits for the purpose of instruction have been made by the nurse.

The weakness of the above system of handling eye diseases among the school children—the prevailing system of all the large cities in this country and Europe—is that neither the Health Department nor the Education Department has full control over the children, and the valuable opportunity to concentrate on education in hygiene is not fully utilized. The exclusion of the infected child from school does not cover the situation. The feeble quarantine which it entails does little else but call attention in a mild way to the contagiousness of the disease and the need for treatment. The necessity for rapid cure in order to limit the period that the child is a carrier of infection and the teaching and enforcement during this period of personal and domestic hygiene are neglected.

The problem in New York City is not an easy one. There is a large foreign population, poor, ignorant, unclean in their habits, many parents willing and glad to use the excuse of "sore eyes" to

have the child remain at home to help in the family work. The children are self-willed and indulged, and when they refuse treatment are not forced by their over-ridden or deceived parents to submit.

Children suffering from folliculosis (up to the past year called "trachoma" by the school doctors) have been permitted to attend school regularly provided they showed proof of being under a doctor's care. Between these children and the school nurse there has been a constant conflict. The child and the child's parents cannot understand enforced treatment where there are no, or so few, subjective symptoms. The child evades the unpleasant and irksome treatment, deceives the nurse, some even going so far as to buy date stamps and stamp their own dispensary cards. Where a particularly rebellious child has refused treatment absolutely, the nurse has had to choose between allowing the child to have his will, thus undermining her authority and that of the school, or exerting her power to exclude the child from school until he submitted—the very object the child frequently wished.

In the case of the excluded children, with their exclusion they pass out of the jurisdiction of the school and the nurse too. Only the actively infectious cases are usually excluded. These children may roam the streets at will, carry infection to all who come in close contact with them (as they frequently do in their play), for the 14 to 16 waking hours instead of the 8 to 10 hours otherwise spent out of school. These children do not necessarily place themselves under treatment; many are glad not to go to school. Girls take advantage of the plea of "sore eyes" to remain at home to help in the housework and are even encouraged by their parents. Boys have been started by exclusion from school on the way to become incorrigible truants. They acquire the street habit.

On the other hand there is the financial hardship to the family when a child approaching 14 years of age is excluded from school and kept from getting his working papers through lack of required school attendance.

After a year's study of the problem, the workers at the Research Laboratory, co-operating with the Division of Child Hygiene, agreed that the exclusion principle was inadequate. They suggested to the educational authorities that experimental

classes for children suffering from infectious eye diseases which ordinarily exclude them from school be opened in one of the most infected parts of the city. In October, 1912, two classes in close connection with a Health Department eye clinic were started in the lower east side at Hester and Allen streets as an annex to Public School 65. One wing of a vacated school building was fitted out by the Department of Education. On the ground floor is the playground, one flight up, two clinic rooms and a demonstration room, and above these, two classrooms and a washroom. The classrooms each seat from 15 to 17 children. The washroom contains a porcelain wash basin with a gooseneck faucet worked by a foot lever, so that the child touches no part with its hands. All washing is done with running water. There is also a bubbling drinking fountain of porcelain worked by a foot lever.

Three days a week a Health Department clinic is held in this building. All children with eye disease from some 60 adjoining public and parochial schools are either sent or brought in squads for treatment by the school nurse. The children who formerly would have been excluded are transferred without delay to the infectious-eye disease classes. The principals of their respective schools are notified by printed post card.

The mild cases of folliculosis are instructed to use boric acid drops at home and are placed under the school nurse's observation. If these cases become marked or show secretion, they are referred back to the clinic. The marked cases of folliculosis are treated at the clinic one or more times a week, chiefly with bichlorid of mercury rubbings. Other cases, such as blepharitis, keratitis, etc., needing treatment report to the clinic as heretofore. The children are given cards with the diagnosis stamped on them for the information of the school nurse who has typed instructions as to what course to pursue.

The children retained in the special classes are treated according to prescribed directions of the clinic doctor twice a day, by the special nurse assigned to duty at the ophthalmia school. Medication formerly left to the child's discretion for use at home is thus given in the school. Now we know that the child receives this treatment. If the child absents himself from school, the truant officer is sent for him.

In addition to actually taking care of the eyes, the nurse in attendance teaches the children personal, family, and social hygiene by talks, demonstrations, and quizzes (see p. 301). In the demonstration room (furnished in simple fashion as a sanitary bedroom), demonstrations on domestic hygiene and the care of the eyes are given to the mothers. The children are taught the value of cleanliness. They are obliged to keep their hands and nails clean, and are taught not to rub their eyes with their hands or wipe them with their coat sleeves or caps. Their hands and persons are inspected daily, and as a reward for cleanliness honor buttons are given them. At the end of the term, the child who has the best marks for cleanliness receives a small manicure set. At the end of the school day, each child washes his own desk and chair with soap and water. This is done chiefly for its educational value.

This school is unique among New York City schools in the matter of cleanliness. The clinic room, the corridors, and stairs are mopped up daily. The waiting-room and schoolrooms are wet-swept daily and scrubbed three times a week. The toilets, banisters, and side walls as high as the tallest child can reach are wiped down daily with a disinfectant. The chairs and desks in the classroom are wiped down with a disinfectant after the children have washed them with soap and water.

All possible precautions are taken to avoid carrying infection from patient to patient. A basin of antiseptic solution is on either side of the doctor so that the hands covered by rubber gloves can easily be disinfected between patients. This is done whether there is visible secretion or not. Medicine droppers are not allowed to touch patients' eyelids, and are always dipped in an antiseptic solution before they are returned to the bottles. Bluestone pencils are not used from one patient directly to another, but are immersed in 95 per cent alcohol for a time before being used again. Pledgets of cotton wrung out of boric acid solution, one for each eye, are given each child to catch the excess of medication and secretion. These are thrown into an enameled pail and burned later.

The nurse visits the homes of the children in attendance and gives instruction to the mothers in their own homes. Printed leaflets in three languages, Italian, Yiddish, and English, giving instructions on the care of the eyes are distributed to every new patient in the clinic (see p. 298).

Two teachers are in charge of the ophthalmia school. The children, ranging from 7 to 16 years of age, are divided into two classes of mixed grades, one of the older and one of the younger children. The children's eyes are protected from strain as far as possible. All work, as far as it can be, is oral, and all reading and writing of very short duration or omitted in severe cases. Regular routine grade work is not made the object of the class. Since the majority of the children spend a comparatively short time in these classes, more stress is laid upon hygiene and the care of their health than on the "three R's." Such work as is given is rather for the purpose of keeping their minds and hands occupied. The school session is shortened, the morning session beginning at 9:15 A.M. and the afternoon session ending at 2:15 P.M., with one hour from 12 to 1 for lunch. One half-hour in both morning and afternoon is devoted to recreation—play in the neighboring park in fair weather, story telling in bad weather. Last year's schedule was as follows:

9:15 to 9:30.	Assembly
9:30 to 10:00.	Treatment and Rest
10:00 to 10:30.	Arithmetic
10:30 to 10:45.	Spelling
10:45 to 11:15.	Recreation
11:15 to 11:45.	English
11:45 to 11:55.	Reading
11:55 to 12:00.	Wash hands and Dismiss
12:00 to 1:00.	Lunch
1:00 to 1:15.	Wash hands and Rest
1:15 to 1:45.	Recreation
1:45 to 2:15.	Hygiene (Monday), Geography (Tuesday, Wednesday, Thursday, Friday).
2:15.	Dismissal to Clinic for Treatment.

Up to June 20, 1912 (9 school months), 312 pupils had passed through the ophthalmia school. The various kinds of cases treated were as follows: acute conjunctivitis (including acute contagious conjunctivitis or "pink eye" and phlyctenular conjunctivitis), chronic conjunctivitis, trachoma, follicular conjunctivitis, syphilitic, tubercular, and phlyctenular keratitis, and neglected protracted cases of blepharitis. When school closed June 28 there were about 25 children in the classes, with 17 ready

for discharge. Only three during the year had been returned to the class after discharge with a recurrence of the disease. The shortest stay in the class was 2 days, the longest, the entire 9 months. Among the latter there were 8 children with post-operative and cicatricial trachoma and pannus or ulcers.

From the health point of view the disease is attacked early, prevented from becoming chronic, and is kept under persistent treatment so that the cure is shortened, while at the same time both children and parents are taught how to avoid reinfection and spreading the disease to others.

We have made the recommendation that the Health Department request the Department of Education to establish one or two more of these special classes in co-operation with a Health Department clinic in the congested districts farther uptown. We feel that with an intelligent nurse especially trained in eye work to oversee the school, there would be no danger of infection to other children if such a class or classes were held in a regular school building. The reason for the belief is based upon the experience with the clinic of Public School 21.

SCHOOL CLINICS.

While we were negotiating with the Department of Education for the formation of the special ophthalmia classes, an opportunity to open an infectious eye disease clinic in Public School 21 was offered. This school is in the congested Italian district somewhat remote from the Health Department infectious-eye disease clinic then located at Gouverneur Slip. As an experiment, this clinic was opened during January, 1912, in a room set aside on the second floor in the school building. We have no special infectious eye-disease class in this building—there is no unoccupied room at present that can be used for this purpose. Clinics are held twice a week during the morning school hours, to which not only the children of Public School 21 come, but also children from the neighboring public and parochial schools. An exceedingly competent nurse was placed in charge to carry out the prescribed treatments twice a day, as in the ophthalmia school at Hester and Allen streets. Children with acute conjunctivitis, phlyctenular conjunctivitis, follicular conjunctivitis, papillary conjunctivitis, in fact all children with conjunctival secretion who in Public School 65 clinic would be placed in the special class, either are excluded temporarily with instructions to report to this school clinic twice a day for treatment or, if secretion is slight, are placed in their regular classroom at a desk somewhat apart from the other children. Directions for avoiding the conveyance of the disease are given to the child and to the teacher. The chair and the desk which the child occupies are washed with antiseptic solution after school and the banisters leading to the clinic room are washed daily with an antiseptic after each clinic is held. Through the exceptional personality and co-operation of the principal of the school, Mr. John Doty, and of the nurse in charge, Miss Schmidling, we have had absolutely no difficulty in keeping the children under control. The excluded cases, which are becoming fewer and fewer in number, report faithfully to the clinic twice a day. The nurse in charge gives instructions to the children in personal and general hygiene, visits the homes and instructs the mothers. In spite of the fact that the school takes care of about 2,500 children and is not kept any cleaner than the average public school in New York City (with the exception of the clinic room which is mopped up daily), we have had very few acute cases during the past two years. We have never traced an infection carried from one child to another in the classroom.

While we do not strongly recommend the clinic in the school alone without the classes for the excluded, it cannot be denied that it works very satisfactorily in this particular school. Without, however, the co-operation of a principal who has a strong personal hold on his pupils and without a thoroughly competent nurse who has a personality that attracts children without loss of dignity or discipline, such a clinic might not be a success. To this clinic the mothers and fathers of the school children, the older and younger brothers and sisters, friends and neighbors, are brought for treatment by the pupils. By treating them and allowing them to come twice a day if necessary, we have gained a strong hold in the neighborhood.

Summer Camps.

Since trachoma is one of the infections which bar children from admission to the fresh-air homes and camps, and since no special provision is made for them, they have no opportunity for a change of environment and food except a rare trip to Coney Island with the family or a visit to a "country relative" in Hoboken or the Bronx. In excluding children from the summer-vacation homes, the broadest interpretation is given to the term trachoma. No child with follicles, whether the eyes be in a secreting condition or not, is admitted. The justice of excluding these cases of folliculosis, now considered benign and non-communicable, is to be questioned. Of course children with conjunctival secretion should be excluded. As I have said above, the children affected come from the poorest homes and the most congested parts of the city; most of them do not receive sufficient or proper food at home.

For a month in the summer of 1911, through the generosity of the A.I.C.P., we were able to send selected children from our clinic to a home set apart for them at Wilton, Connecticut. The result was most satisfactory. A nurse and a helper were with the children so that their treatment was carried out daily. Following this demonstration, it was hoped the Health Department would construct a permanent summer camp for trachoma and infectious eye diseases, but funds were not obtainable and the matter is in abeyance.

In the summer of 1913 the *New York Globe* made an appeal to its readers and through their generosity enough money was collected to send 29 children to a farm at Cuddebackville, Orange County, New York, for the whole 10 weeks of vacation. The isolated location and the sanitary arrangement of the farm were well adapted to the demands of such a summer home. A Health Department nurse and a volunteer helper were with the children all summer and the routine treatment was carried on twice a day as in the special classes in New York. The doctor visited them at intervals. The children were out in the open air all day long, and bathed daily in the river. Each child had certain duties, such as making her or his own bed, helping in tidying up the room, and washing his or her own clothes. The children were housed in three dormitories, each in an individual bed, with individual bags holding the clothes, at the foot of the bed, and, of course, individual soap and towels. They washed in the running water of a small runlet in the open air. Cleanliness of the person and general behavior were marked daily on a bulletin posted in the hall and had a splendid effect in keeping up the eagerness to excel. All of these cases were chronic and had been under observation for a long time. Of the children who spent all 10 weeks there, none gained less than 5 pounds in weight and a few gained as much as 10 pounds. Those who were there a shorter time gained from 3 to 6 pounds each. As far as the eye conditions were concerned, while in several cases the improvement

was marked, in the majority we did not find any more noteworthy improvement than occurred in the city under similar constant daily treatment. We feel, however, that the summer work is important and worth while and ought to be made permanent for these reasons:

1. During the summer vacation, there is no one to urge the child to undergo treatment. He neglects himself and what has been gained from the winter's treatment is lost in the summer. Some of these children who were in the country have been under our observation for more than 3 years: their condition before they came to us was chronic and has remained so. Each fall they return to school with a relapse.

2. The knowledge that faithful attendance at the clinic will mean a vacation in the country in the summer is a strong incentive to keeping under constant treatment. Since they are barred elsewhere this is their only chance to get to the country.

3. While miraculous improvement does not accompany the sojourn in the country, the improvement of their general health, the better opportunity for the teaching of personal hygiene while in residence and the prevention of relapses play an important (tho not brilliant) part in the program of prophylaxis.

4. Furthermore, neglected and running the streets of the city, these children are nuclei of infection.

Since the institution of the special ophthalmia classes, school clinic and summer camp, there has been without doubt a distinct improvement of the eye conditions in the lower east side of New York City. The large majority of patients now treated at the clinic since the opening of the second year of the special school (1913) are those of folliculosis, blepharitis (which is either on the increase or else more care in picking out the cases is taken by the school nurse), and pediculi of the eyelashes. This latter condition certainly seems to be increasing, especially in one limited section of the lower East Side.

The fresh, acute cases are seen early, are segregated, and usually run a short course. Only an occasional freshly developed case of papillary conjunctivitis has been seen since the above measures have been instituted, while between 1910 and 1912, 320 cases of papillary conjunctivitis were treated by us. The folliculosis cases seem milder; none has been sent to the hospital for infectious eye diseases for the operation of expression since October, 1912.

2. PERSONAL HYGIENE AND "FOLLOW-UP" WORK.

Alice Kurtz
assisted by
Ella Lipsky, Mary Schmidling, and Theodora Herzig.

Early in the work of the Research Laboratory group on the preventative treatment of trachoma, home visits to patients attending our clinics were begun with several objects in view: (1) to examine and record the eye condition of other members of the family in order to determine the degree of home infectivity and to advise treatment; (2) to secure a more complete history than clinic conditions permitted; (3) to investigate the environment for the purpose of determining helping causes; (4) to give instructions, both verbally and by demonstration, for the prevention of the spread of infection, and (5) to follow up the work to be sure that the instructions were being carried out.

I. Investigation.

The first question investigated was that of infectivity in the series of cases here presented. This is considered under two heads, Helping Causes and Home Infectivity.

helping causes.

At the outset inquiry was always made as to what was regarded by the family as probable source. Public baths, street playing, particularly ball playing, the schools, public and parochial, injuries, the visiting of relatives in Europe having trachoma, and also the eye clinics themselves were frequently given as the source of sore eyes. Many children complained of never having had sore eyes until some time after being sent to the clinics by the school nurse. Other suggested sources were "catching cold," a children's school in the country, libraries, school books and desks, and banisters in houses where there had been an epidemic.

Out of 117 families marked for general cleanliness only 4 are marked double plus, which shows that even minimum health standards in the home are not an easy matter, and only 35 are marked plus, indicating cleanliness moderately good.

Of course the question arises: Is it necessary to depend upon evolution here for results? We feel that this may be answered emphatically in the negative because of the hopeful results in habit-formation obtained by us in our ophthalmia school.

While in community or family work discouragement is nearly always present, the work with the children in the special school is encouraging for the friend with patience and perseverance who forgets iron-clad discipline—so called—and wins the confidence and friendship of the child.

Improper nourishment.—Among the most serious hindrances to good physical condition in these children is unwholesome food. Almost every child in every family gets at least one penny a day because he cries or kicks for it. Many, of course, get more. Many parents are literally afraid to refuse pennies and continue to dole them

out even when the child has no appetite, rarely eats breakfast, and runs to the candy store as soon as he is out of bed in the morning. One small boy of six, still covered with day-before dirt, was seen at the breakfast table with sister and mother coaxing him to take an appetizer of whiskey to aid in the swallowing of a piece of fried fish, some coffee and bread. Nothing of this breakfast was eaten, but it was freely fingered. The visitor was told that he never had an appetite altho he ate a large amount of candy. Coffee and rolls for breakfast are almost universal from the baby of two to the grandmother who looks 80 but is nearer 50. Lunch is frequently a "snatch" meal of a piece of dry bread or bread and butter or often something bought at a push cart. Some effort on the part of teachers and visitors is made to substitute the buying of fruit for sweet stuffs. Some mothers prepare a lunch for the school children. The cleanest homes and the best foods are in orthodox families or the families with the highest ethical standards. The evening meal is the one substantial meal of the day and is usually chicken or other meat, with vegetables boiled in the soup pot. Lack of proper nourishment, and an abnormal amount of sweet stuff were found almost everywhere.

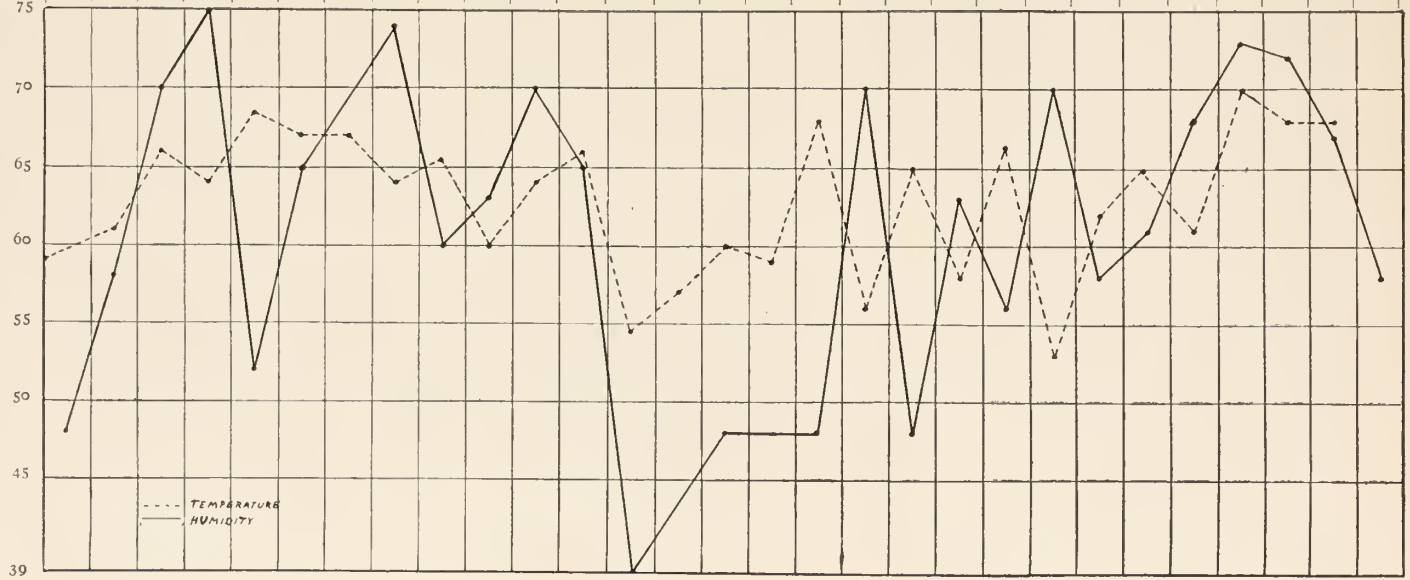
Overcrowding.—Of 116 families, on the basis of allowing 8 persons to 5 rooms or 5 persons to 3 rooms, the number of families overcrowded was 67 and on the basis of allowing 1 person to 1 room (including kitchen) the number overcrowded was 111. Of the 5 families not overcrowded, only one family had more rooms than individuals, 3 persons to 5 rooms. Only 2 families out of 116 enjoyed the same number of rooms as there were individuals in the family. The other two least crowded households had, respectively, 8 persons to 6 rooms and 8 to 7 rooms.

Upon recommending a separate bed for one small boy, the visitor was informed that it could be managed. A few days later the boy came to the visitor and said he was not going to sleep that way any more, he did not like it; that he was afraid of rolling off. The separate bed had been made on the top of an upright piano! Owing to the noise and general disturbance in one family of 11 living in 4 rooms, the older son had to put off his home study until after eleven o'clock at night. Owing to the overcrowding of the rooms, to the noise, light, and disturbance on the part of their elders, the children do not manage to get to sleep much before ten and frequently are kept up later.

Light.—The measurements of light, temperature, and humidity were undertaken under instructions from Professor Winslow, of Columbia University, who has decided that the results shown in the light experiments are suggestive of wrong method, and so they are left out of his report. The method used, however, showed at least the wide variations as, for example, the difference between 46.5 in a dark kitchen, and 81 at the front window on the first floor of the same apartment. An attempt was made to measure the light in the most used room of each of the 50 families visited. In this study no definite connection between dark rooms and trachoma has been shown.

Temperature.—The temperatures shown on Chart 1, taken in December and January, indicate that there is not much overheating. In 41 families no temperature was lower than 53° F. Between 53° and 55° F. there were 3 families; between 56° and 60° F., 12; between 61° and 65° F., 12; and between 65° and 70° F., 14. There was no case over 70° F. Thus, out of 41 families, 33 had 66° F. or under, 15 had 60° F. or under, and 3 had 55° F. or under. However, may not the desirability of some of these low temperatures be questioned, especially when noted in connection with poor food, or little of it, ill health, and insufficient clothing? Of course, in respect

Number in room	3	4	2	2–4	6	4		7			2–6	6	4	4	7	7	2	6	3	5	8	4		2	2	3	9		5
Heat or cooking-steam	None	None	None	C	R	R					R							R C						R C		R C			R C
Kind of room	S	K	K	K	K	K	D R	K	K	K	K	K	K	D R	D R	K	K	K	K	K	K	K	K	K	K	K	K	K	K
No. of family	1	2	3	4	5	6	7	8	6	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29



S = sitting-room; K = kitchen; D R = dining-room; R = range; C = cooking.

CHART 1.—Showing range of temperature and humidity in 29 families.

to both temperature and light it is largely only the mother and babies who would really suffer, as most of the others in the family are at home a comparatively short time, except when in bed.

Humidity.—The range of temperature and humidity in 29 homes may readily be seen on Chart 1. The drop from air temperature, or difference between wet and dry thermometers, was measured by means of the Sling psychrometer and the actual humidity was obtained from the relative humidity table of Queen and Company, Philadelphia.

Toilets.—Out of 158 families only 13 had toilets in their own apartment. According to law there must be one toilet to every two families. On nearly all floors there are 4 families and 2 toilets. Many of our patients have 8–13 in one family, 2 water closets for 32–52 people! Taking the average number in the families as 7, it means that in one house, on one floor of 4 families and 2 toilets, there are 14 men, women, and children to one toilet; that is, in the whole house of 6 floors there are 170 persons using the 12 water closets in this *one* house 365 days of the year, going through narrow, dirty, and dark halls, stumbling over stairs, filthy and dark, and over stray cats and children. This is only in *one* house. This one house is massed up against another so much like it that the laws in regard to light and air are followed barely in the letter and not at all in the modern human spirit. One of the consequences of this overcrowding is that the children run frequently to the street for urination. Naturally the necessary washing after the use of the toilet is seldom if ever carried out. In the home apartment there is water—frequently only cold—in the kitchen only, and beside the kitchen sink hangs the family towel. The use of individual towels and washcloths and much soap and water is almost nil. Most of these people have not been taught the importance of personal hygiene. The sanitary requirements of the city are for the most part carried out except perhaps in regard to care in avoiding odors. Stale odors and others were marked in many cases in the halls and toilets. With the exception of a few houses—one in markedly bad condition—the toilets were fairly clean, at least superficially. The space occupied is very limited.

Employment.—The largest number of fathers seem to be employed in sewing or pressing clothing or in selling. Of 60 families, 21 of the mothers act either as janitress or help at selling in store or at stand, while but 2 do home work—aside from housewife's work. When sometimes the workers of the family, including mother and father, leave as early as 5 o'clock in the morning and the care of the children is left to the oldest child, it may be considered as little short of marvelous that the mother, or one of the older daughters, creates a semblance of order and cleanliness once a week, out of most chaotic and filthy conditions. Everywhere an effort is made to have a clean house on the Sabbath.

Income.—Altho somewhat eager to note the income of these families it was found to be impossible in the time at our disposal or without special investigations looking to that end. While the questions of income and rent were put with as much tact as possible, much keener ingenuity was displayed in avoiding the answer. The means of existence ranged from none at all—where the workers were on strike, or where the principal occupation was "looking for a job," or because of sickness or seasonal occupation—to bank accounts of fair size. These latter were not acknowledged but inferred, and in one case proved, where the mother, who needed expert care quickly and had been sent to a hospital by the visitor, was refused as a suitable charity case

by the city Department of Charities. It was found through letters from a bank that this family had an account of at least $2,000, while anonymous warnings placed the savings at from $8,000 to $10,000.

Summary.—From the foregoing it will be seen that the chief causes regarded as contributory to the spread of chronic conjunctival affections are the crowded and unwholesome conditions of tenement-house life, which involve contact of normal children with those infected, and which lower the general physical condition and resistance of the child. These conditions are based upon insufficient income, inertia, and ignorance. Parents frequently bear too heavy burdens in the care of a large family of young children during strikes, dull times, and "off" seasons in seasonal occupations. The cases of deliberate indifference and neglect on the part of the parents are few.

HOME INFECTIVITY.

As our school clinics were in but two districts the children belonged, with comparatively few exceptions, to two races, the large majority being Jews and most of the others, Italians. Out of 174 cases the number of children known to have been born abroad was about 40. The two series of studies on infectivity, one by C. R. Gurley (p. 285) and the present one, contained a majority of families common to both, but a small number peculiar to each. The estimate of infectivity was, however, made upon a somewhat different basis in the two cases. For each series were selected families in which at least one member had trachoma and in which were at least several other individuals accessible to infection. In the first series only those members of the family were counted as infected from the trachoma individual whose cases were diagnosed as trachoma. In the estimate which follows, however, cases diagnosed as doubtfully trachoma as well as those of trachoma (papillary conjunctivitis with follicles), were counted as originating from the individual having the condition called trachoma.

Tables were made including 64 families representing an aggregate of 577 persons. Of these families with an average of 9 individuals each, 27 had home infection very probably and 2 had home infection probably. In the 27 households showing home infection, there were 211 individuals, of whom 68 were infected (see Table 2). Calculating upon the basis of one member in each family having been infected from outside, we have 41 out of 184, as the number of home infections, or 22 per cent.

The 27 families in which home infection occurred had an average population of nearly 8, yet the average space to a family was $3\frac{1}{3}$ rooms, or about $2\frac{1}{3}$ persons per room.

While the percentage of home infectivity resulting in cases diagnosed as trachoma seems to be low, the infectivity of certain cases of acute conjunctivitis in the home and among playmates has probably a very high percentage. Thus in a family of 8 all had "pink eye" with the exception of the two older sisters who slept together. No one knew how the infection was caught, but it was found that the baby of two years had

sore eyes first. This child had been playing with another child in the house who had had "sore eyes," but who was now well. The boarder and all except the two older daughters of the family used one towel. These two daughters slept together and used one towel in common, but kept it in their bedroom.

TABLE 2.

SHOWING PROBABLE HOME INFECTION.

NUMBER OF FAMILY	INDIVIDUALS IN FAMILY	NUMBER INFECTED		NUMBER OF FAMILY	INDIVIDUALS IN FAMILY	NUMBER INFECTED	
		Adults	Children			Adults	Children
1	8	1	4	16	8		1
2	8		2				1(?)
3	9		1	17	9		1
			1(?)				1(?)
4	11		4	18	6		3
5	8		2	19	7		3
6	5		2	20	11		2
7	11		1	21	6		1
			1(?)				1(?)
8	7		2	22	5		3
9	6		3	23	8		1
10	8		4				1(?)
11	9		3	24	9		2
12	7	1	1	25	8		1
13	7		3				1 (Conj.)
14	7	1	2	26	10		2
15	4		2	27	9		1
							1(?)
	115	3	38		96		27

Total number of individuals exposed to infection, 184 (211 minus 1 in each family regarded as source).

Total number of individuals infected, 41 (68—27) or 22 per cent.

TEACHING.

In teaching, emphasis has been placed (1) upon immediate and follow-up instruction of the individual child and members of the family in all cases of secretion, and (2) upon preventive work with other members of family.

In the winter of 1910–11 in the clinic in the Research Laboratory, each child on entering the clinic was observed by one of Dr. Williams' assistants and careful instruction given. Owing to the large number that soon came to the clinic, it was found impossible to give individual instruction and group instruction was supplied to those who came one-half hour to one hour before clinic. A hygiene club was formed and minute instructions given; the members promised to come to clinic with, and to keep, clean hands, neck, ears, and noses, and to ask for the use at home of separate pillow, separate towel, separate soap, to wash under running water if possible, and in general to carry out the details of the home instructions given them.

HOME VISITING.

In home visiting, in order to avoid implications of carrying infection from family to family, it was important that the nurse-visitor should not touch with unprotected hands the sore eyes of the child. Rubber gloves were worn and the hands were dipped in an antiseptic solution immediately before touching the lids. Thus sterile gloves or cotton wet in boric acid solution alone touch the lids.

To get results, unless much more time could be given in the individual home, the demonstrations in cleansing the eyes in the home were discontinued except in cases demanding immediate attention and in which the mother could not come to clinic or demonstration rooms.

As a consequence home visiting excluded demonstration of cleansing of eyes in the home, except in rare cases, and included gaining friendly relations with mother or both parents. The history of "sore eyes" in family and neighborhood was obtained; methods of utilizing home material and space for carrying out the suggestions given at clinic were demonstrated. Mothers were invited to the special demonstration room. Prevention and control of infection in the home were taught with constant emphasis upon cleanliness and the infectivity of secreting eyes. All members of infected families were kept under observation.

That this work of preventive teaching may accomplish wonders is illustrated by the case of a center of infection in Broome Street. One child, sent to the clinic by the school teacher, had not appeared at the clinic. On notification, the special nurse went to the house and interested the mother in bringing her child to the eye clinic. No other children in the family had an eye infection, but there were several cases of sore eyes in the neighborhood. Six children were then called upon, all of whom named one child as having the first sore eyes in the neighborhood. This boy, who had been a case at our own clinic, was now well, but there were 7 cases from this particular playmate source. The playmate source of infection, whether at home, at school, or on the street, seems to be one that needs special attention.

A great deal of effort has been expended in striving for some control over family habits, the majority of homes visited possessing a family towel. Suggestions as to the necessity of separate towels, face cloths, soap, burning of eye cotton, faithful cleansing of hands and eyes, were met with a ready acquiescence—seldom fulfilled—or with a hopeless shrug of tired shoulders. In some few families, a special effort was needed to help the families themselves in finding separate towels. Fairly good towels would be pulled forth from a weird collection of rags and a nail was found somewhere and hammered into the desired spot. One or two of the families, however, after the whole family had become infected, had learned certain details of care by bitter experience, and so realized the importance of our efforts to instruct them. Thus Mrs. ——— asked why all this effort and teaching of mothers and children had come so late. "Why," said she, "did you not try to do this seven years ago, when we first had sore eyes? I myself, many a day, have sat on one of those benches and told mothers to take care of their children's eyes. I am no bacteriologist, I cannot even write my own name, but seven years ago, I thought the mothers ought to have been taught and ought to have had the leaflets you are giving today."

In spite of the usual inertia, the word "catching" was in nearly all cases an open sesame to interest and friendliness. The difficulty of changing the attitude or actions of some of the families may, however, be observed in the case of Harry K. For two years this family was visited frequently, the mother attended demonstration classes, yet Harry became worse. Even coming to the clinic several times a week did him no permanent good. He was sent to the hospital until the secretion, which constantly poured from his eyes, was taken care of. On improvement he was returned home, but was soon again in the same old state. This was a simple case of home neglect and mental or perhaps physical inertia. His mother, who had been almost constantly ill, was sent to a hospital and after a sojourn of several weeks she came home, moved

into lighter rooms, and in general took more interest in the life of her family. But the real change became apparent when this child was made to come to the special school regularly. He was treated two or three times a day or as often as the doctor thought necessary. He was made to become as clean as was possible at the school. His nose, eyes, and fingers were under constant supervision. His mother actually co-operated when she became stronger, by insisting upon his attendance at school without the help of the truant officer. She also kept him in fairly clean blouses, something that had been unknown before. It is believed that she even made him take his medicine at home. Harry was always dull, stupid, and obstinate, his physical handicaps making him more or less lifeless. Since he had been out of school for months and as he could scarcely see, he sat in the backyard most of the time playing with the baby. The life in the school, however, and the daily walks to the park with other children soon changed him into a much livelier boy.

CLINIC INSTRUCTIONS IN PREVENTION OF INFECTION.

Such a simple operation as washing the nails and arms has to be taught time after time in minutest detail and with never-ending patience. Much attention was paid to cleanliness of nails, without aid of brush or other nail apparatus, getting the soap—preferably "green" soap—well into nails and using a finger nail of the other hand as a cleaner. Talks at the same time were given on the purpose of the nails, and on drain pipes and the special need of, and particular pleasure in, having the individual soap and towel. Altho washing the hands before meals is a religious and health law with the majority of our patients, the methods in use leave much to be desired, as the family towel mutely but eloquently shows.

[The English "Home Instruction" leaflet, printed in three languages.]

DEPARTMENT OF HEALTH.
The City of New York.

Home Instructions Regarding Care of "Sore Eyes."

GENERAL.

Before attempting to cleanse the eyes, wash hands and finger-nails, using warm water, and plenty of soap, particularly in nails.

Wash hands as thoroughly **after** touching the eyes.

Wash under running water if possible, but if basin is used, scrub basin with soap (or soap powder), hot water, and brush before and after using.

Have separate towels for each person in family.

Have separate washcloths for each person in family.

Have separate pillow-cases for each person in family.

Have separate handkerchiefs for each person in family.

Those with sore eyes should not sleep in same bed with others in family.

After using, pillow-cases should be aired, in sun if possible, and then covered or kept from contact with other pillow-cases.

After using, towels should be aired, in sun if possible, and so hung that they do not touch one another.

All towels, handkerchiefs, pillow-cases, washcloths, used by person with sore eyes should be changed, washed, and boiled as often as possible, but at least twice each week.

It is important to keep child's nose clean by blowing nose at least twice a day regularly, morning and night.

To clean nose, apply some vaseline in nostrils, on cotton wadding wound about a toothpick or burned match.

Have windows opened at night. Give simple food to eat. See that bowels move daily.

Keep child's fingers and finger-nails clean.

TO WASH EYES.

First wash hands as directed above.

Have boric acid water and clean absorbent cotton (from drug store) or clean (that is, boiled) soft, old white rags.

Use one piece of cotton at a time, and on one eye only; wipe away secretion (matter) carefully from eye, and eye-lashes; burn cotton at once.

Wash eyes every hour, or oftener if necessary, while child is excluded from school.

As long as child is under treatment at dispensary, eyes should be washed at least five (5) times each day.

TO PREPARE BORIC ACID WATER.

Buy boric acid powder. Add one (1) heaping teaspoonful of boric acid powder to one (1) cupful of hot water. Boil in covered pan. Put in clean bottle when cool. Shake thoroughly. When cool, sediment, if any, will sink to bottom of bottle. Do not shake again. USE WHEN COLD.

TO USE MEDICINE.

First wash hands as directed above.
Then wash eyes as directed above.
Have clean medicine dropper.
Have piece of moist cotton to catch overflow, do not use handkerchief.
Have child's face upturned and eyes opened.
Drop medicine in corner of eye, nearest nose.
Have child close lids and roll eyeball.
Wash away overflow with moist cotton, do not use handkerchief.
Burn cotton at once.
Wash hands and finger-nails again.
Clean medicine dropper.

TO CLEAN MEDICINE DROPPER.

Remove rubber top. Boil for about one (1) minute, both glass and rubber parts in water containing washing soda. Rinse in clear water when cool.

Keep all eye utensils clean and dry, and free from dust.

FREE CLINICS FOR CONTAGIOUS EYE DISEASES

Department of Health

580 East 169th Street, Bronx.
341 Pleasant Avenue, Manhattan.
P.S. 21, Prince and Mott Streets, Manhattan.
P.S. 65B Annex, 78 Hester Street, Manhattan.
330 Throop Avenue, Brooklyn, N.Y.
124 Lawrence Street, Brooklyn, N.Y.
1249 Herkimer Street, Brooklyn, N.Y.

In some contradistinction to the difficulties found in certain families the real helpfulness and interest shown in many families may be mentioned. In each of the four, out of 117, families showing unusual cleanliness at each visit, separate towels were already in use and separate beds in two. A high degree of intelligence with bodily health also existed here.

MENTAL ATTITUDE.

Rating the mental attitude exhibited by parents is rather difficult but an attempt was made to show with plus and minus marks the attitude and personal responsibility of the parents. In the chart kept of these points the percentage of indifference is remarkably low, only four cases on the part of the mothers showing inertia or indifference. Considering the extraordinary difficulties under which the majority of these mothers labor, together with their physical ailments, the personal responsibility and interest shown is very high. Of these 51, the fact that 13 or about 25.5 per cent showed marked initiative is very encouraging for future home work. Under receptivity, the majority are marked fair.

Of the three difficulties, overcrowding, too much work (including illness), and poverty, overcrowding stands out as the most positive, aided largely by a general lack of order.

DEMONSTRATIONS AND MOTHERS' CLASSES.

Owing to the difficulties of teaching asepsis in the home and the ineffectiveness of attempts by the mother to follow verbal instruction, it seemed important to establish a demonstration or teaching center for the mothers and children.

With the most active and cordial co-operation of Miss Kittredge, founder of the Association of Practical House-Keeping Centers, more thorough teaching work was started at two of the model flats of the Association, one on Henry Street in the Jewish district, and the other in the Italian quarter. Because of lack of time, the work in the Italian quarter was later discontinued but the model flat on Henry Street was in use until the old school at Hester and Allen streets was fitted up for special class and clinic work. In this latter building a demonstration room is now provided. The demonstration room contains 2 beds, spread as if ready for use, a table, several chairs, and a washstand with basin, pitcher, and soapdish, surrounded by a folding screen. All the furniture is white-enameled and the basin and pitcher are of white enamel-ware (unbreakable). On screw-hooks in the framework of the screen hang the separate washcloth and towel for each member of the supposed family. By means of a long, horizontal strip of tape attached to the washable material with which the screen is covered, the comb and toothbrush of each individual are provided with a place, as in a rack, near the washstand.

During the winter of 1911 the mothers and their infected children were invited to the model flat where the kitchen and sitting-room were used for the purpose of demonstration. The advantages of having a kitchen much the same as the kitchens in the district were evident when it came to disposing of cotton that had touched the eye and when the arrangement and care of basins and other utensils, soap, and individual towels were considered. In the cold weather hot cocoa and biscuits were served in the model dining-room to our mothers and children.

In the demonstration room or model flat the actual "Home Instructions" (p. 298) were carried out. Demonstrations were given in the care of hands and nails,

washing of eyes, method of dropping in medicine, care of utensils, handkerchiefs, towels, and pillow cases, burning of eye-cotton, making of boric acid solution, etc. The most compact arrangement for having separate beds and the best method of caring for a number of separate towels in a small space were shown in the sitting-room. To these were added, at the special class, simple experiments, and talks and quizzes in preventive hygiene, with practical work by the young members of the classes. The demonstrations showed just what is possible in the home. The "trachoma nurse" then visited the family, after detailed instructions had been given to the children and mothers, to see that separate towels were used.

Special Class Instructions.

Upon the establishment, through the co-operation of the Board of Education and the Health Department, of a special school of two classes and an affiliated clinic for children excluded from the regular school on account of contagious eye diseases (see p. 288), the work of instruction in hygiene was transferred to it.[1]

In connection with the "special class" or ophthalmia school are given demonstrations and talks on prevention of contagion and on personal hygiene. Every child who enters the clinic except in "rush hours" is persuaded thoroughly to clean his hands and nails while a little lecture is given on the connection between diseases, especially his eye trouble, and dirty hands and towels. Occasionally one or two have an "afraid from it," but the combination of a little tale about microbes, a little ball of green soap just for himself, with a separate paper towel, and a comparison between his own black nails and the white ones shown him, usually wins over even the most obstinate. In the clinics, also, is shown to child and parent or visiting relative, the proper methods of using medication and how to avoid touching the eyes with the fingers.

As a single example of what may be accomplished through the special class may be mentioned the case of a boy who showed little or no result after a year and a half of effort on the part of the visitor to the home, but quite marvelous improvement after a few months of the constant supervision afforded by the school.

In connection with the classrooms of the school the washroom is the center for individual instructions in reasons for coming to the class, how to take care of the apparatus shown—the washbasin and drinking fountain have pedal attachments—how to wash hands and nails, and what to do at home in order to help himself and to prevent others from getting the disease. The necessity of appearing at school every morning with clean hands, neck, and ears, and just how and when to wash eyes and face, are emphasized.

With certain cases attending the special class, special intensive work was done. It is interesting to note that of 51 children who received individual instruction, more home visiting than could be done upon patients as a whole, and clinic demonstrations, all but 6, or 88 per cent, are marked on the hygiene chart as showing positive effort on the part of the children to put into daily practice the instruction received in class and home. The six are marked fair; no one is marked as failing.

[1] The great difficulties were largely overcome by the faithful watchfulness on the part of the teachers, Miss Mansion and Mrs. Smith, under the direction of Mr. Bulkley, principal of Public School 65. Without their interested and sympathetic co-operation, the efforts at habit formation would have been largely unavailing. It was necessary to counteract many lifelong tendencies by patient reiteration of the desired substitutes.

The Committee on Prevention of Blindness, of the Russell Sage Foundation, has very kindly lent us large educational screens depicting the dire results of the family towel and other causes of trachoma. The study of these screens is part of the education of each little pupil-patient. One screen, an awful example of the roller towel, causes shivers when the new patient is asked if he thinks that is the proper thing to use against his face. It seems so easy to agree that this towel is a carrier of disease—or rather that it might become so if in use—just as the screen says, but it is so hard to see the blackness of the home article.

The teaching of general cleanliness was also impressed upon the children through the care of their desks. Each was given a piece of cheesecloth and a basin. With these and copious water and a "ball" of green soap, every afternoon before leaving, each desk was washed. The cloth and the basin were kept clean by the child who was intrusted with them. This work did not affect the routine of school cleaning by the school helpers, however, which included the washing with carbolic solution, at regular intervals, of the desks and all parts of the school touched by the children's fingers.

Half-hour talks on hygiene were given twice a week to the senior class. These talks included some reference to the anatomy of the normal eye and very slight reference to the general anatomy. Only drawings and prints were shown. Practical talks were given and practical results were expected along hygiene lines. Outlines for the daily care of the body were required to be written out, and quizzes, many and searching, were a real delight to the nurse-teacher as mental results seemed to come with much promptitude and ease, whereas physical results were at times discouraging. Finger-print impressions were taken on agar-agar and showed many brilliant results in growths of vari-colored bacteria and moulds. The interest of the children in workable hygiene in this school seems to point the way to wonderful possibilities. The same amount of time was given to the little ones and they showed just as much interest in the agar experiments, and were desperately serious in the tale of the stove that "went out" because it had too much food thrown in and could not get rid of the waste, and in the comparison with the stove that could do so much for people when it had a good fire burning in it. They were familiar with stoves. The effective screens of the Committee on the Prevention of Blindness were used here too.

Honor-roll.—To each of those who carried out the teachings in cleanliness for a month, keeping day by day up to a certain standard, was given, with due ceremony, an enameled button on which was printed "Clean Hands, Clean Habits, Clean Homes." Besides this, all children were daily marked for cleanliness.

On passing in review the many hindrances to a healthful and improving home life for our patients, several suggestions present themselves. Instead of shorter school hours why not longer school hours—not at a desk, but longer hours under the control of the school, spent in play in the open air, in rest, in domestic science and manual training? In connection with their instruction on the prevention of infection, both boys and girls could be taught how to wash and boil clothes properly, and the necessity of so doing. The boys were always interested in the possibility of making a

screen such as the one used in the "model" demonstration flat and the arrangements necessary for keeping individual towels. Such interests might be utilized in their manual-training work. Some of the children now come to school at any hour from 7:30 A.M. on, only too glad to get away from an unwholesome atmosphere of 9 persons in 3 rooms or 11 persons in 4 rooms. Perhaps with this lengthening of the hours spent under intelligent supervision will come legislation advancing the age limit at which a child may leave school. He leaves school now at the most unstable period of life.

The opportunities of teaching clean habits and other health habits have been mentioned. In this respect a decided advantage might be gained could other visitors to the home, especially the ethical teacher and Hebrew reader, be enlisted to co-operate in giving the never-ending reminders.

The lunch hour could prove a splendid source for suggestive teaching. In most schools and homes it is now a time of trial for all concerned.

Is it necessary for all the children to have tuberculosis before the open-air school comes into general use? Probably all of these children under observation in our clinics are anemic or at least subnormal.

CONCLUSIONS.

The burden of the large reduction in the number of bad contagious eye cases should be laid to several factors, the most prominent being probably the different methods followed in the eye clinics. The factor regarded as next in importance is the marked development of "follow-up" work in connection with the clinics, including special attention to "first time" acute cases.

The follow-up work should include at least the following aspects:

1. Teaching, in the home of every infectious case, of the ways of avoiding the spread of infection, and of how to control the condition in the member affected.

2. Securing, through home visits and through demonstrations and talks in connection with the clinics or the special class, of more hygienic habits of life.

CO-OPERATION.

Throughout the work we have received much cordial co-operation from many who have been interested in its progress.

Dr. R. T. Wilson, superintendent of Health Department hospitals, gave us valuable advice and material assistance in the arrangement and furnishings of our clinics and demonstration room.

Mr. Sparre, of the Brevoort Mission House, and Miss Kerr, of the Department of Health, have taken much pains in arranging for summer outings for our children; while Mrs. M. M. Marre and Mrs. Weil aided financially in sending some of the children for summer vacations.

A friend of the clinic arranged for a splendid Santa Claus celebration in our Christmas season.

Mr. John Doty, principal of Public School 21, who suggested the value of the school clinic, helped in many other ways. We also owe a great deal to him and to Mr. Kidd and Mr. Bulkley, of Public School 65, for their active aid, as we do to Miss Mansion and Mrs. Smith.

To Professor Agide Pirazzini, of the Bible Teachers Training School, we owe the translation of the Home Instructions into Italian, and correction of the proofs. Miss Mishulow, of the Laboratory, very kindly undertook the translation into Jewish, while through the overtures of Mr. Frank, superintendent of Beth Israel Hospital, the Jewish translation was typewritten and printers' proofs corrected.

To Miss Kittredge our thanks are due for the use of unusually favorable demonstration rooms; while it is impossible to enumerate all that we owe to the Committee on Prevention of Blindness.

Mrs. Weil interested a group of young Jewish girls in supplying the "Honor Roll" buttons, which were given to those who made an effort for "Clean Hands, Clean Homes, Clean Habits."

Miss Sharp, of the Beth Israel Hospital, took a personal interest in those members of our patients' families who were referred to the hospital or to its Social Service Department. Mr. Frank, superintendent of the same hospital, aided in many ways.

Many philanthropic and social agencies cordially co-operated in the care of our families; for example, the New York Department of Charities and Corrections, the A.I.C.P., the United Hebrew Charities, and the Beth Israel Hospital.

The Educational Alliance, the College Settlement on Eldridge Street, and the Clark Neighborhood House were interested in getting in touch with the boys and girls, through classes and clubs and motion pictures.

Where destitution or some particular need, such as milk or eye-glasses, was found or suspected, the United Hebrew Charities or other relief agency or interested hospital was notified and cheerfully responded. It may be added here that where Big Brothers or Big Sisters were thought to be part of the treatment of any member of the family, the Educational Alliance, the University Settlement, and other Neighborhood Houses promised their aid. The one important difficulty here was the over-demand for help along this line.

In one instance the Committee for Prevention of Cruelty to Children most kindly sent a visitor to give aid and counsel to a mother distracted by her oldest boy's midnight habits. The Educational Alliance sent tickets to their "moving pictures" in order to get some of our difficult boys interested in going to the Educational Center.

IV. CURATIVE TREATMENT INCLUDING VACCINE TREATMENT OF INFECTIOUS EYE DISEASES.

H. W. Wootton and Anna I. von Sholly

ASSISTED BY

Caroline R. Gurley, Olga Povitsky, and Percy Crane.

In regard to our curative treatment of trachoma and its allied conditions or of those affections of the conjunctiva which are characterized by the formation of follicles or by hyperplasia or by both, and which, by their continuance or exacerbations, may be considered as likely to eventuate in cicatrization and involvement of the cornea, we may state that, in general, the mechanical and

surgical methods and the drugs employed have been those described in every textbook. The only new treatment tried by us is that of vaccines in certain selected cases which will be discussed later.

We wish, however, to emphasize particularly the following points in our handling of the series of cases described in the previous paper (p. 295): (1) the large number of cases which were treated from or near the beginning of the disease; (2) the frequent change of treatment in the subacute and chronic cases; (3) the absence of drugs and surgical treatment in a large percentage of cases formerly supposed to need them.

The applicability of the various methods of treatment to the four groups of conjunctival affections already described is shown in the following brief statements of the results obtained.

Folliculosis (follicles scattered over an otherwise apparently normal conjunctiva).—In mild cases no treatment was used except boric acid solution (3 per cent) at home and an insistence upon observance of rules of personal hygiene. These cases were sent to the clinic once a month for examination or sent by the school nurse within that time if inflammatory symptoms supervened. The great majority of these cases, so far, have at present normal conjunctivas, and the others are practically normal (Table 1). In more marked cases bichlorid of mercury (1–500) rubbing with cotton swab was employed once a week. Marked improvement followed even one rubbing in some of these cases. In a few of the more chronic cases sulfate of copper stick was infrequently used. In this series of some 2,000 cases of folliculosis observed for from 2 to 4 years, we have not yet found it necessary to use expression. If the operation of expression is performed, it should be clearly explained to the patient that the disease attacked will not eventuate in deterioration of vision and that the operation itself may be followed by unpleasant consequences to both conjunctiva and cornea.

Follicular conjunctivitis (follicles scattered over a non-thickened inflammatory conjunctiva).—In these cases, if the superadded inflammation is acute and attended with secretion, argyrol, 20 per cent, or 1 per cent silver nitrate solution was first used. Later, bichlorid of mercury was rubbed on the inner surfaces of the lids. With Morax-Axenfeld diplococcus infection, sulfate of zinc (0.2 per cent) was used. In the few sluggish cases the bichlorid of mercury treatment was alternated infrequently with the bluestone pencil. The great majority of these cases recovered without the last-named treatment. The hygienic treatment at home and in the schools is insisted upon in all of the cases.

Papillary conjunctivitis (formerly called acute trachoma).—Argyrol, 20 per cent, or silver nitrate solution, 1 per cent, was used in the beginning in these cases, when there was usually more or less marked secretion. Later the bluestone pencil alternating with tannic acid (40 gr. to an ounce of glycerin) was employed. From time to time in the more chronic cases all medicinal treatment (except the home boric acid washing) was suspended for a variable period. After this, a certain number of the cases seemed to respond more favorably to the renewed medicinal treatment. Simple hygienic treatment for several months would probably be sufficient for a great number of these

cases if we could have them under hospital care as Cohen and Noguchi had their cases which recovered in an average of 4 months after only boric acid and cold compress applications.

None of our patients with papillary conjunctivitis has been operated upon.

Old trachoma (cicatricial formation with or without pannus).—In our series of cases among the school children we have as yet had no cases passing into the cicatricial stage tho several have had pannus. Among our pannus cases only 8 have developed the pannus after being seen by us. Two of these left us and all of the other 6, none of whom has been operated on, now have clear corneas, except one who has a narrow rim of inactive haze over the upper part of each cornea. These cases, treated medicinally in the usual way—some of them by vaccine—are being carefully watched for further developments.

In the adult, of course, the condition of cicatrization, with its danger of probable supervention of corneal involvement, is complicated by the fact that the patient is usually a wage-earner. As Boldt very truly states, our estimation of the value of a method of treatment must depend largely upon its applicability. These chronic cicatricial cases as a rule, and in New York City almost invariably, are found among the working classes, who have neither the means nor the inclination to pursue any palliative or expectant plan of treatment, and for economic reasons it is in these cases, in the opinion of one of us (Wootton), that excision is pre-eminently indicated, and that in all cases in which the cornea has actually become involved, excision should be performed at once. Excision, however, does not always mean cure since a certain percentage of individuals in our old pannus cases have shown a continuation of the pannus in aggravated form after the operation of excision.

Acute catarrhal conjunctivitis.—When our laboratory workers found that hemoglobinophilic bacilli of the same type were found in many cases of acute, subacute, and chronic conjunctivitis, we made special efforts to follow up in our treatment all of the acute cases of conjunctivitis in the schools and in the homes of the children in order to eliminate this form of infection from chronic cases. We cannot yet tell whether the fact that we now have very few of the chronic papillary or follicular conjunctivitis cases is due to these preventive measures.

Vaccine treatment.[1]—The following is a tentative report on the results obtained from the use of subcutaneous doses of various vaccines in a few cases of eye infections at the Health Department clinic for infectious eye diseases. The favorable results obtained with tuberculin in phlyctenular and ulcerative keratitis confirm the reports of other investigators. The rapid cure of styes and infected meibomian cysts treated with staphylococcus vaccine accords with our expectations. The use of streptococcus vaccine for pannus and interstitial keratitis, suggested by Dr. Williams, is, as far as we know, new. Since it has been used less than three months, no positive statements can be made. The marked improvement, especially in Cases 1 and 2 which had previously been very recalcitrant, is most suggestive and encouraging.

Streptococcus Vaccine.

pannus.

Case 1, 11 years old. Duration of sore eyes not known but under our observation almost 2 years. Her conjunctivas during this time presented deep-seated follicles on both upper and lower lids, at times with and at times without inflammatory symptoms but no corneal involvement, until 8 months after we first saw her.

[1] For the report given here of vaccine treatment in these cases Dr. von Sholly is responsible.

October 29, 1912: Pannus developed in right eye. The von Pirquet test made at this time was negative. Daily treatments of atropin, hot applications, and ung. hydrargyri flav. ox., 2½ per cent into conjunctival sac, were given, with either bluestone or corrosive sublimate (1–500) rubbings twice a week. After little less than 3 months' treatment, the right cornea became clear again.

March 4, 1913: Pannus developed in both eyes but was more marked in right.

May 27, 1913: A Wassermann test was negative. The same treatment as above was instituted but very little improvement was noted.

June 30, 1913: Child was sent to our country camp but after 10 days returned on account of a death in the family. Until school opened, September 16, 1913, she was seen only twice and had marked pannus in both eyes covering half of cornea, and pronounced photophobia. All over her face there was also a scaly papular eruption which had been present all summer.

September 29, 1913: Twenty-five million streptococci (stock vaccine) were injected.

October 3, 1913: Fifty million were given. After the second injection, the pannus disappeared from the left cornea, leaving a very faint scar. The right cornea showed a marked recession of the pannus. After the third injection a week after the second, there remained only the lightest haziness of the extreme upper part of the right cornea.

November 11, 1913: After the fifth injection, the pannus had entirely disappeared.

December 6, 1913: Both corneas continue clear except for a scarcely perceptible scar in each. Streptococci were found in the cultures made from the conjunctival curettings. Curiously her skin condition improved also and now her skin is absolutely clear.

Case 2, 15 years old. "Sore eyes" 3 years.

February 12, 1912: Operation (expression) New York Eye and Ear Infirmary.

July 10, 1913: Came to our clinic with a papillary conjunctivitis of both eyes, more pronounced in right, marked and very active pannus covering more than half of right cornea and a small scar of a former ulcer on left cornea. The von Pirquet test was negative. In spite of this, she was given, in increasing weekly doses subcutaneously for 7 weeks, tubercle bacillus emulsion, beginning with 1/10,000 mg. At the same time atropin, hot applications, and yellow oxid of mercury ointment 2.5 per cent in conjunctival sac, were used daily. Bluestone was used at intervals. At the end of this time the very active symptoms had cleared up, but the pannus of upper half of right cornea persisted.

September 6, 1913: During the last week of the tubercle B.E. injections, the old ulcer on the left cornea became active. The T.B.E. injections were stopped.

September 16, 1913: An inoculation of 25 million streptococci (stock vaccine) was given. This was continued in doses increased by 25 millions once a week. In 16 days after the third injection the ulcer on left cornea was healed, leaving a very light scar which still persists (December 6).

October 23, 1913: The pannus of the right eye receded slowly after the sixth injection. The infiltration of the cornea, from being moderately dense, had become very light so that the iris could be clearly seen, and the blood vessels running in from the top had become reduced to only a few of the larger ones.

October 30, 1913: A mixture of streptococci (175 million) and *Sta. pyogenes aureus* (500 million) was injected.

November 1, 1913: There was a striking improvement noted. Only a little infiltration of the cornea was left at the top with one or two small vessels running into it.

November 11, 1913: The right cornea was almost normal except for a slight rim of cloudiness at extreme top.

December 6, 1913: There is still this small hazy rim at the top. The thickening of the palpebral conjunctiva has decreased so that the left palpebral conjunctiva, tho still reddened, has become thin enough to allow the palpebral blood vessels to show to a certain degree.

Case 3, 33 years old, married.

December 21, 1911: Came to the clinic with a cicatricial condition (non-operative) of both palpebral conjunctivas and sluggish pannus of both corneas. Duration of eye trouble 4 years or more. She was treated in the usual way with hot applications, atropin, yellow oxid of mercury ointment, and bluestone. She has continued under constant observation.

March 3, 1912: She had an acute exacerbation with intense vascular reaction of pannus first in right and then in left eye. The right cleared up under the above treatment.

July 27, 1912: While pannus was still acute in the left eye, patient was given 50 million polyvalent hemoglobinophilic bacilli. The injections in increasing dosage were given weekly for 9 weeks.

September 28, 1912: Both corneas had improved to such an extent that there was only a very light cloudiness of the upper fourth.

October 15, 1912: While these injections were still being given an active acute pannus started up in right cornea and on November 9, 1912, in left. The injections and local treatment were continued and in two weeks all active symptoms were gone, leaving the corneas slightly cloudy again at the top.

June 5, 1913: A mild exacerbation of pannus in left cornea appeared lasting only a few days. Mrs. L. was at this time pregnant. On August 21, her baby was born. Eight days later she appeared at the clinic with a very severe reaction in the left eye. There was a dense opacity of the whole cornea and marked ocular and ciliary injection.

September 4, 1913: Six days after onset, 25 million streptococci were injected. In 5 days improvement was noticeable,. By the seventh day, on which second injection was given, the improvement was marked, and by the ninth day the acute symptoms had subsided. After the fourth injection the corneas were clear except for slight blurring above and after the eighth injection on November 1, 1913, both corneas became quite transparent with no show of pannus. Streptococci, staphylococci, and xerosis bacilli were found in cultures.

Case 4, 10 years old, a thin, pale, and undernourished child.

March 4, 1912: "Follicular trachoma" and pannus in both eyes. Under daily local treatment and injections of vaccine of hemoglobinophilic bacilli, begun October 15, 1912. In December, 1912, 9 months later, the pannus in left had become less, and after another month the pannus had returned and covered upper half of right cornea and about upper third of left with a small active ulcer on left also. The child was sent to our country camp for 9 weeks. He gained 5 pounds and his general condition was much better, but the pannus, altho a little less dense, was present in both eyes.

September 11, 1913: A combined vaccine, streptococcus (25 million) and *Sta. pyogenes aureus* (200 million), was injected. Streptococcus vaccine alone was then given for 3 doses (weekly) and again the combined.

October 30, 1913: After the third injection of the latter, the pannus of left eye was entirely healed, leaving a very light scar, and the pannus of right was much less. The last injection was given November 16.

November 18, 1913: A few fine ulcers appeared on both corneas.

November 20, 1913: Tuberculin B.E. 1/10,000 was given.

November 25, 1913: The corneas had cleared and were more transparent than they had been since he was under operation. Left cornea shows only small scar and right the merest rim of infiltration at the top. After the second injection of tuberculin the cornea looked in good condition.

December 9, 1913: After the fourth injection, the pannus had returned to both eyes, now covering fully half of each cornea. Streptococcus injections now will be substituted for the tuberculin.

Case 5, 40 years old, married. Duration of disease 8 years. Combined excision of Heisrath 5 years ago. Canthotomy last year.

December 13, 1910: Came to clinic with pannus of both eyes, trichiasis. Under our observation and local treatment her pannus has receded and relapsed at varying intervals. She was given the hemoglobinophilic bacillus vaccine from May 2, 1912, to April 12, 1913, at first regularly and later rather irregularly. For a time there seemed to be marked improvement but later no effect. For a time she was under the care of another dispensary.

October 4, 1913: She returned with a very severe active vascular keratitis involving whole right cornea and part of left. She was given empirically 25 million streptococci. For three days after this injection she was somewhat worse, then began to improve the day following the second injection.

November 4, 1913: Fourth injection was given and on November 20, fifth injection. On this date, her right cornea was almost clear; there were no active symptoms and there was only a slight cloudiness of left cornea.

December 5, 1913: Both corneas were clear except slight blurring at top and there were no active symptoms.

Case 6, 17 years old. Came to clinic with severe double pannus. Could not work. Had a combined excision of palpebral conjunctivas and tarsal cartilages. Under daily local treatment and two injections of staphylococcus vaccine, he improved so much that he has not returned to clinic.

SPECIFIC KERATITIS.

Case 1, 15 years old. Double keratitis of several years' standing; dense opacity of both corneas.

December 12, 1912: Came to clinic. Von Pirquet test negative. Wassermann positive on January 4, 1913, and on September 16, 1913. Had two injections of salvarsan on February 8, 1912, and May 27, 1913, respectively. Wassermann still positive almost 4 months after second injection of salvarsan. Took mixed treatment by mouth.

June 30, 1913: Sent to summer camp for 9 weeks, when she improved considerably. In September her corneas presented a very light infiltration, and she could read and do her school work.

October 18, 1913: An injection of 25,000,000 streptococci. On the following day there was an intense ocular and corneal reaction, almost like that following the salvarsan. It lasted 4 days and left an increased cloudiness in the cornea.

November 11, 1913: Almost 3 weeks after the first injection the second injection of streptococci was given with no reaction. After the third injection following a week's interval, both corneas became clearer than they had been since her first visit to us. Five injections in all were given and now there are transparent areas in corneas with small faintly blurred areas.

TUBERCULOUS KERATITIS.

Case 1, 10 years old.

June 11, 1912: Came to clinic with dense mottled bluish-gray infiltration of left cornea with injection, photophobia, and discomfort. Palpebral conjunctivas normal. Under usual local treatment, she showed slight improvement by July 2.

July 16, 1912: A fresh ulcer with active symptoms appeared and by July 26, there was a distinct ectasia of cornea. The child again improved slightly under local treatment.

August 20, 1913: She received a blow on the left eye and appeared with a complete dense infiltration of cornea with ocular and ciliary injection.

October 8, 1912: Von Pirquet test gave a moderate reaction. Three days after this test was made, a fresh crop of small ulcers appeared in lower part of left cornea.

October 18, 1912: Tubercle bacillus emulsion 1/10,000 mg. was injected. This was given weekly, increasing each dose by 1/10,000 mg., until January 10, 1913, when 1/1,000 mg. was given. After the third injection the cornea had cleared sufficiently to show the pupil; by the sixth injection the cornea was transparent except for a few tiny macules.

January 10, 1913: The cornea looked so well that the tuberculin was stopped.

April 15, 1913: A small fresh ulcer with local injection appeared on the cornea and tuberculin B.E. was again begun at 1/10,000 mg. One week later the ulcer was healed, leaving a tiny scar. Injections were continued, however, and the dosage carried to 1/100 mg. on September 19, 1913.

August 5, 1913: The cornea was quite clear.

December 5, 1913: The last record shows that the cornea is still transparent except for a few very small almost imperceptible blurs which are seen only on close inspection. The patient has gained a good deal in weight and general condition since the T.B.E. was started.

Case 2, 9 years old. Sore eyes for 8 months.

May 18, 1912: Entered clinic with eye condition similar to Case 1, cornea ectatic. Gave a very pronounced reaction to the von Pirquet test both at site of vaccination and locally in eye. Within a few days after the von Pirquet test was made, there was a noticeable improvement in the eye before the injections were begun.

May 28, 1913: T.B.E. 1/10,000 mg. given.

June 8, 1913: The ectasia of cornea had disappeared and opacity was much less dense.

June 27, 1913: The lower part of cornea alone was hazy with scattered more dense flecks.

November 6, 1912: There was only the slightest blur on cornea. Patient had begun to be irregular in attendance.

January 7, 1913: Two fresh ulcers developed but left after a few days under tuberculin. The child was not seen then until March 6, 1913, when she reappeared with several small ulcers on upper part of cornea. 1/10,000 mg. T.B.E. given.

March 13, 1913: She had improved and was given a second dose of 2/10,000 mg. Recovered and remained away from clinic.

May 10, 1913: She appeared with tiny ulcer just above pupil. Otherwise her cornea was quite clear. An injection of tuberculin was given. She has not reappeared at the clinic.

Case 3, 13 years old.

October 22, 1912: Came to clinic with several small ulcers on corneas of both eyes. "Sore eyes" for 2 years. Had photophobia and blepharospasm.

December 20, 1912: Von Pirquet positive. Eyes showed same improvement after test as in Case 2.

December 24, 1912: Tuberculin B.E. begun. Increased gradually to 5/100 mg. by June 24, 1913.

January 14, 1913: Both corneas were clear, no photophobia, no blepharospasm.

January 28, 1913: A fresh ulcer appeared but cleared up rapidly.

March 11, 1913: Another small ulcer broke out which healed rapidly.

April 4, 1913: Both corneas were transparent.

May 25, 1913: Last ulcer appeared.

October 31, 1913: Both corneas were clear and have continued so. Only irregular injections were given after June, none at all during the summer. A few follicles have appeared on palpebral conjunctivas since under our care. They were probably stimulated by the local treatment of atropin, etc.

Case 4, 10 years old.

April 1, 1913: Came to clinic with a severe vascular keratitis covering whole right cornea with dense infiltration. Left cornea had old scars. Duration of eye trouble two years. Child had a healed tuberculous spine. Von Pirquet test positive.

April 11, 1913: Tubercle B.E. was begun 1/10,000 mg.

April 15, 1913: A slight ectasia of right cornea was noted.

May 13, 1913: There was much improvement and no active symptoms after 4 injections.

June 6, 1913: Right cornea was transparent except for a few macules.

June 28, 1913: Last injection 2/1,000 mg. was given. Both corneas were clear except for light blur over centers.

September 2, 1913: The condition was the same.

Case 5, two and one-half years old.

July 31, 1913: Came to clinic with left eye showing a complete deep lilac-colored infiltration of whole cornea, thickly vasculized and totally obscuring iris and pupil. Cornea looked as if it had ruptured and healed. Child had a tuberculous knee. Mother had died of tuberculosis.

August 2, 1913: Von Pirquet test positive.

August 15, 1913: Tuberculin injections beginning with 1/20,000 mg. B.E. were started. After the second injection the vessels disappeared from cornea and cornea took on a bluish-gray tinge. The process had gone too far to have any hope of saving the eye but the child's general condition and knee condition have improved wonderfully. From a peevish, fretful, pale child, he is now happy and cheerful, with a good color, and gets about well with a brace.

December 2, 1913: He was receiving 3/1,000 mg. T.B.E.

Staphylococcus Pyogenes Aureus Inoculations.

ACUTE CONJUNCTIVITIS.

Case 1, 50 years old. *Sta. pyogenes aureus* found in cultures.

September 18, 1913: Came to clinic with intense ocular injection, injection palpebral conjunctiva, with much discomfort, profuse watery and some mucopurulent secretion. At the end of two weeks' local treatment with silver nitrate solution argyrol, boric acid solution given daily by nurse at clinic, there was practically no improvement.

October 2, 1913: 1,000 million *Sta. pyogenes aureus* (stock vaccine) were injected. Two days later the eye was well.

INFECTED MEIBOMIAN GLAND.

Case 2, 40 years old. Abscess of meibomian gland ruptured into conjunctival sac and set up an acute conjunctivitis. Patient was a teacher anxious to return to work. Case was seen on third day, argyrol and boric acid advised. On the following day, the eye appeared about the same and staphylococcus vaccine (500 million) was injected into arm. The improvement on the following day was striking and the second day after the injection the patient returned to her work.

About a dozen other cases of infected meibomian glands, some of which had ruptured and some not, have been treated similarly with immediate and permanent results.

About 27 cases of styes, many of them recurring ones, have been given staphylococcus injections, varying from 1 or 2 to 6 injections, with very satisfactory results.

V. MICROSCOPIC AND CULTURAL STUDIES.

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ASSISTED BY

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Having soon reached the conclusion mentioned in the review of the literature (p. 262), that, notwithstanding the voluminous publications on the subject of the bacteriology of conjunctivitis, only a few comprehensive bacteriologic studies have been reported; and at the same time being impressed from our early microscopic studies with the resemblance in morphology and staining reactions of the trachoma, or Prowazek, inclusions to dense groups of small bacteria, we determined to make, in connection with the clinical work, as exhaustive a cultural and microscopic study of our cases as was practicable with the facilities at hand and with our present knowledge of the characteristics of pathogenic microorganisms.

Technic.—When we began our cultural studies each case showing any inflammation of the conjunctiva, after being clinically examined and recorded as given in Sec. II, was subjected to the following microscopic and cultural examinations. A light, but thorough curettage was made with an eye curette, usually over the upper tarsal and *cul de sac* regions. The curetted material was used chiefly for the films, but occasionally some of it was cultured. The films were made by cover glass pressure, were fixed immediately in methyl alcohol, C.P., and were stained over night in Giemsa's solution (see Gurley and Chase for details of method of staining). Other stains, Gram's, methylene blue, fuchsin and methylene blue mixture, etc., were used from time to time.

The majority of the cultures were made as follows: Over the freshly curetted conjunctival surface was rubbed a sterile cotton swab which was placed immediately into a 10×1 cm. tube containing a mixture of veal broth and rabbit or horse blood (10:1), about 3 cm. deep. The tube was then sent to the laboratory where further cultures were made. This preliminary blood-broth culture acts in two ways: (1) immediately, as a diluent in cases where many organisms are present, and (2) after growth in the incubator, as an enricher, especially for the group of hemoglobinophilic bacilli and for the gonococcus. From this blood-broth tube stroke cultures were made on plain veal agar, ascitic-glucose-agar, and blood-(rabbit or horse) veal-agar, and in deep tubes of these media containing agar, inoculated while fluid, allowed to harden and then covered with more melted agar and, when solid, with albolene. Cultures, including the original tube of blood-broth, were kept at 36° C. and examined in from 18 to 24 hours, when a second series of cultures was made from the original blood-broth culture, diluted when necessary. Fishings and films were made from plates, and all cultures were returned to the incubator. In 48 hours fishings and films were made again and all cultures were studied for varying times. The deep tube cultures were observed for anaerobes and were examined in from 4 to 10 days. These deep tube cultures were discontinued after a short time since we found no organisms growing in the depths that did not grow in the blood-broth tubes.

After we had examined in this way a number of conjunctivitis cases, we concentrated in cultural work on those diagnosed clinically as active trachoma, as suspicious, and as doubtful cases or beginning trachoma. From time to time through overfull clinics a number of these cases did not receive this complete examination, but such cases were recorded and examined later if they continued to be of interest. Only a few folliculosis cases were thus examined, and many of the acute catarrhal conjunctivitis cases cleared up too quickly for a later culture study.

In a number of cases before cultural studies were begun when we were interested chiefly in the morphologic examination of the trachoma, or Prowazek, inclusions, we studied only smears; and in a number of cases later, especially of acute contagious conjunctivitis, where our chief point for a time was to determine the

prevalence of the group of hemoglobinophilic bacilli as well as the relation of our hemoglobinophilic strains to the influenza bacillus and the Koch-Weeks' bacillus, we studied only cultures.

Eight hundred and twelve cases were examined by cultures, 500 cases by direct smear (chiefly for Prowazek inclusions), and 400 cases by both cultures and direct smears.

The 812 cases examined by cultures were obtained from the following places:

Cases from New York City school children and their families:	
Borough of Manhattan (lower East Side)	607
" " Bronx (upper East Side)	38
" " Brooklyn	9
New York City Eye and Ear Hospital	21
Blackwells Island	19
Ellis Island	16
Home for Feeble Minded	79
Kentucky Mountains	13
Miscellaneous	10
	812

We see by glancing at Table 3 that the hemoglobinophilic bacilli (excepting staphylococci which were present in practically all conditions in varying numbers) were found most frequently in these cases, particularly in the cases diagnosed as papillary conjunctivitis (at first called acute trachoma) and acute conjunctivitis. The first strains of this group that we isolated from cases diagnosed clinically as trachoma were so extremely small and coccus-like that we thought we were dealing with a new variety, but we soon found that the different strains varied somewhat in size, and that the same strain in subsequent culture generations varied in the same particular. When we found that all the strains isolated from our acute conjunctivitis cases, including all cases of clinically typical "pink eye," as well as from our subacute and chronic conjunctivitis cases, were strictly hemoglobinophilic, we came to the conclusion that these bacilli must be classed, at least provisionally, as influenza bacilli, and much of our subsequent study was directed toward determining whether these strains might be divided into distinct species or varieties of the influenza group or whether they all belonged to the one species, *B. influenzae*.

We recognized further that from a clinical standpoint, too, it was important to determine the relationship of our strains to the Koch-Weeks bacillus, since so many of our acute cases were clinically typical "pink eye," or acute contagious conjunctivitis, and since several of our early cases passed from this condition to that of papillary conjunctivitis showing trachoma inclusions. So we set about systematically hunting for the classic Koch-Weeks bacillus. For this purpose we chose a series of early marked typical "pink eye" cases before treatment and submitted them to our microscopic and cultural technic. In all of these we obtained in

TABLE 3.

Showing Clinical Diagnoses and Cultural Findings.

Diagnoses	Total No. Cases	Hemoglobinophilic B.	Pneumostreptococcus Group	Str. pyogenes Hemolyzing	Gonococcus	Morax Axenfeld	Tetragenus	Yeast	Diphtheria	Typhoid Colon Group	Sta. Pyogenes	Xerosis B.	No Growth	M. catarrhalis
Old trachoma	59	9	14	....	....	1	....	....	....	1	35	25	....	.....
Papillary conjunctivitis	237	147	60	2	5	6	2	5	....	4	190	95	1	.....
Follicular conjunctivitis	145	49	6	1	0	8	....	....	....	4	130	65	....	.....
Acute conjunctivitis	271	204	95	4	8	2	....	....	2	5	250	156	2	3
Folliculosis	45	0	0	....	....	.....	....	....	....	.....	19	15	23	.....
Other conditions	55	15	18	....	....	.....	....	....	....	3	32	31	....	.....
Total	812	424	193	7	13	17	2	5	2	17	656	387	26	3

our original tubes and plates abundant and practically pure growths of a strictly hemoglobinophilic bacillus similar to our other strains. We fished from many different colonies in each plate, but no classic Koch-Weeks bacillus was found. The original smears showed bacilli sometimes larger (the Koch-Weeks bacillus?), sometimes smaller (the influenza bacillus?), but only abundant hemoglobinophilic bacilli grew. That we may have missed a bacillus answering to the classic Koch-Weeks bacillus is possible. One has only to remember the history of the demonstration of the Bordet-Gengou bacillus to realize how unwise it is to draw hasty conclusions from resemblances between morphology in cultures and that in direct smears. In these pertussis studies at first only hemoglobinophilic bacilli were isolated from pertussis cases. Finally the distinct variety, *B. Bordet-Gengou*, was grown. It may be that we in the same way have not yet been able to grow

the classic Koch-Weeks bacillus. While we do not believe this, all we can positively say is that in this large series of cases of clinically typical "pink eye" we have secured in direct cultures abundant growths of a strictly hemoglobinophilic bacillus, which answers to the description of the influenza bacillus rather than to that of the classic Koch-Weeks bacillus. And this fact has led us to agree with those who consider the Koch-Weeks bacillus a strict hemoglobinophile, indistinguishable so far from *B. influenzae.*

The following tests were made in order to help determine the characteristics of our strains.

Hemoglobinophilic tests.—We went very minutely into the question of the hemoglobinophilic property of our strains. Most of the experiments relating to this point were made by Miss Mishulow. Besides the preliminary attempts to cultivate these strains on blood-free media, we made repeated attempts from time to time on a larger number of the older strains, all with negative results. We tried to accustom the strains to growing without blood by growing them in gradually diminishing amounts of it. We found that tho they grow well in amounts up to 1–1,000 (horse blood), the growth stops after a variable number of culture generations when this amount is lessened. Horse hemin crystals[1] in veal agar or broth in dilutions of 1 to 100 and 1 to 1,000 gave no growth after the third culture generation.

Horse serum, obtained by drawing the blood in sodium citrate and, after allowing the red blood corpuscles to settle, pipetting off the clear supernatant fluid, gives variable results. Some sera allow growth for many generations and others for only a few. In the former case it cannot be denied that the positive result is probably due to the presence of a minute amount of hemoglobin. When we remember that in 1 to 1,000 blood dilution it is only detected by a more delicate test than the spectroscope, e.g., Adler's benzidene test, we may realize how easily, by the method used for obtaining the serum, hemoglobin in amounts large enough to allow growth might be present and might escape detection. The positive cultures claimed for the Koch-Weeks bacillus in media containing "rich hydrocele or ascitic fluid" may be explained by the presence of such a minute amount of hemoglobin.

Symbiosis tests.—In connection with the attempts to make these strains grow without hemoglobin, we carried on a number of so-called "symbiosis" tests, i.e., we grew certain strains on blood-free agar and broth coincidentally with other bacteria. The results obtained on blood-free veal agar are condensed in Table 4. All these tests were repeated with practically the same results. In blood-free broth Strains 636 and 351 (hemoglobinophilic) grew well with xerosis bacillus for 12 culture generations. B.I. 1 (influenza bacillus) was negative on the sixth generation in the first test, but had a growth on the eighth in the second; while B.I. 2 (influenza bacillus) failed to grow beyond the seventh culture generation in this fluid medium. As plain and blood-veal agar and broth were used as controls in this work we had a further test of the hemoglobinophilic property of these strains. Many individual tests were made of symbiosis on plates as well as in tubes, which bore out the results shown in Table 4, namely, that all the organisms tried, except *B. xerosis*, *B. diphtheriae*, and the

[1] Isolated by Mr. Atkinson.

gonococcus, interfered with the growth of these strains. This is entirely contrary to all statements in regard to the influence of the staphylococcus on this group of organisms, and we cannot explain the apparent contradiction. It is true that on plates, if the staphylococci are few, isolated colonies of the hemoglobinophilic bacilli on the first culture generation from a blood-agar medium or on blood-agar media may be larger in the neighborhood of staphylococcus colonies, but only incidentally so, we believe. Attempts at symbiosis growths with cultures of staphylococci, xerosis bacilli, or diphtheria bacilli killed at 60° C., the first two for one and one-half hours and the last one for 15 minutes, were negative after 3–7 culture generations. The practical fact learned from this study is that when many staphylococci are present coincidentally with hemoglobinophilic bacilli in a case of conjunctivitis, the former may interfere with the isolation of pure cultures of the latter.

TABLE 4.

Growth of Hemoglobinophilic Bacilli in Symbiosis on Blood-free Veal Agar.

Strains of Hemoglobinophilic Bacilli	Sta. pyogenes		Staphylococcus + Streptococcus		Xerosis		Xer. + Streptococcus		Streptococcus		Gonococcus		Diphtheria		B. Hoffmanni	
	Gen.	Growth	Gen.	Growth	Gen.	Growth	Gen.	Growth	Gen.	Growth	Gen.	Growth	Gen.	Growth	Gen.	Growth
636	1	−	1	−	20	+	3	−	4	−	19	++	12	±	2	−
351	.....	.....	.....	.....	20	++	2	−	2	−	20	++	12	±	5	−
BI. 1	1	−	1	−	5	−	3	−	4	−	20	+	11	±	4	−
BI. 2	.....	.....	.....	.....	14	++	.....	.....	.....	.....	.....	.....	12	± ?	2	−

In order to try to inhibit the growth of the staphylococci in mixed cultures and allow the growth of hemoglobinophilic bacilli, Churchman's gentian-violet test was made by Miss Wilson and Miss Mishulow. Details of these tests are as follows: Dilutions, ranging from 1–50 to 1–1,000, of a saturated aqueous gentian-violet solution were made in sterile blood-broth. These dilutions were distributed into small test tubes, and inoculated with practically equal quantities of 24-hour agar cultures of Strain 636 (hemoglobinophilic), *Sta. pyogenes aureus* and xerosis bacilli in pure culture and in mixtures. Controls of each were made in blood-broth. Blood-streaked plates were made of these cultures immediately after inoculation and also after ½, 1, 2½, 24, and 48 hours' incubation at 36° C. These plates were examined after 24 hours', 48 hours', and 3 days' incubation. On the whole the best results were obtained after 1 hour's incubation in the gentian-violet-blood-broth.

The results, as indicated in Table 5, show that altho the growth of the gram-positive organisms was not completely inhibited in the mixtures which gave a good growth of the hemoglobinophilic bacilli, it was restricted to a considerable extent. Thus in the controls of the mixed cultures the plates had a heavy growth of *Sta. pyogenes aureus* after 24 hours' incubation and were completely overgrown after 48 hours; whereas, on the plates of the gentian-violet cultures, even when there was a good growth of *Sta. pyogenes aureus*, it was not heavy and the colonies were discrete. Two plates of the mixed cultures of Strain 636 and *Sta. pyogenes aureus* on the 1–50 dilution, one made immediately after inoculation, and the other after one hour's incubation, had a pure growth of hemoglobinophilic bacilli in the center and a good

growth of *Sta. pyogenes aureus* on the margin of the blood zone. Also several plates of a pure culture of *Sta. pyogenes aureus* on 1–50, 1–100, and 1–200 dilutions showed a similar growth. Evidently the growth of *Sta. pyogenes aureus* was inhibited in the center of the plate by the presence of a small quantity of gentian-violet carried to the plate with the culture, since the loopful of material is placed in the center and then radiated from the center.

TABLE 5.

One-Hour Gentian-Violet Broth Emulsion.

Culture	636 + Sta. + Xer.			636 + Sta.		636 + Xer.		636	Sta.
Control	±	+++	++	−	++	+	+	+	++
G.V.Dil. 1–50	±	−	−	−	−	±	±	−	−
1–100	±	−	+	±	++	−	±	±	±
1–200	±	−	±	−	+	±	±	±	+
1–400	+	+	+	+	+	+	++	+	±
1–500	+	±	±	+	+	+	+	+	+
1–600	+	+	+	+	+	+	+	+	+
1–800	+	+	±	+	±	+	+	+	+
1–900	+	±	+	+	+	+	+	+	+
1–1,000	+	+	±	+	+	+	+	+	+
3-day gentian-violet plate all dilutions	−	−	−	−	−	−	−	−	−

± indicates very slight growth. ± indicates slight growth.

The same dilutions of gentian-violet used in the blood-broth tests were made in liquid agar at a temperature of 55° C. and plates poured immediately. When they were solidified a drop of rabbit's blood was put in the center of each plate. Cultures of hemoglobinophilic bacilli (636), *Sta. pyogenes aureus*, xerosis, in mixtures and alone, as before, were inoculated into blood-broth and from these the gentian-violet and control plates were made. The growth on the gentian-violet plates was negative on all dilutions, while the control plates showed growth as indicated in Table 5.

We found by this study that by using the first method described, i.e., inoculating a blood-broth-gentian-violet medium with material containing hemoglobinophilic bacilli in mixed culture with staphylococci, we might expect material aid in isolating pure cultures of the former. Unless all aids possible are used in a cultural study of these cases, we feel that a certain percentage of infections with hemoglobinophilic bacilli as well as other microorganisms may be missed.

Virulence tests.—In connection with the study of antibody production by Dr. Povitsky a comparatively few tests were made of the degree of virulence possessed by our strains for rabbits and guinea-pigs. The results indicate on the whole a similarity to strains of hemoglobinophilic bacilli isolated from other parts of the body. That is, recently isolated strains are irregularly virulent for rabbits and guinea-pigs on intraperitoneal inoculation, and the virulence which is rapidly lost on artificial cultivation may be markedly increased for a species (guinea-pigs) by successive animal passages.

Eight monkeys, 4 macacus species, and 4 dog-faced baboons, were inoculated into the conjunctiva with 48-hour cultures of 8 different strains. One out of the 8 (a macacus) showed, after repeated inoculations, a subacute conjunctivitis, during the course of which a few superficial follicles developed.

Agglutination tests.—A strain of hemoglobinophilic bacilli from an Ellis Island case (a deportable trachoma case from Poland) produced agglutinins of 1 in 2,400 for itself and one from our public-school "trachoma" cases, but not for two other public-school strains or the strain from influenzal meningitis. Other strains, however, from public-school cases produced agglutinins for this meningitis strain.

The only inference we can draw from these studies is the same as that drawn from studies on agglutination in hemoglobinophilic bacilli from any other source; namely, that in the hemoglobinophilic group of bacilli there are distinct varieties as regards agglutinin production, but that these varieties seem not to be related to any definite set of morphologic characteristics or clinical symptoms.

The *complement-binding test* has not yet given satisfactory results. This is chiefly because no antigen has been obtained that would show a specific fixation, apparently because of the serum content of the culture media used. Recently, by growing organisms on media containing a minimum amount of heated blood, i.e., coagulated blood-agar (1 part of blood in 500 parts of agar, at 90° C.), we are apparently beginning to make good antigens. It may be that, with further testing, the fixation of complement phenomenon may help in a study of these strains.

During the attempts to obtain a good antigen, we have tried various methods of obtaining abundant growths of our strains in a medium containing no substances that might interfere with good antigen production. Thus we again tried to lessen the serum content of our medium by gradually diminishing the amount of blood used, but we found, as before, that beyond 1–500 dilution we were likely to get scant growths and beyond 1–1,000 no growth. Even in the dilution of 1–500 the growth is not always abundant unless the blood is added to very hot agar. A temperature of 90° C. seems to be sufficient to destroy something in the blood that is deleterious to growth, for Dr. Povitsky has found that growth is very luxuriant on media made in this way. On salt-free blood-agar, growth is as abundant as on the mixture of ordinary veal-agar and blood.

So far, however, neither the complement-binding test nor any other test tried has helped us differentiate our hemoglobinophilic strains from *B. influenzae* or the Koch-Weeks bacillus. Until we can make this differentiation we must consider all of these organisms as possibly identical in potential pathogenic power; just as we consider a pneumococcus isolated from the eye similar in possible pathogenicity to a pneumococcus from any other source.

RELATION OF HEMOGLOBINOPHILIC BACILLI TO TRACHOMA INCLUSIONS.

In studying closely the morphology in cultures of these bacilli, we were struck by the fact that they frequently grew in more or less dense clumps of extremely minute and irregular coccoid forms. This led us to the conclusion that possibly they formed the trachoma, or Prowazek, inclusions, and when we found that these bacilli and the inclusions were found coincidently and repeatedly in so many cases diagnosed as acute papillary trachoma (see Table 6, p. 320), the possibility became a probability and we

proceeded to study the morphology of the cultures more minutely. Each culture was kept under observation for many days. Each day spreads were made from the same culture and stained by Giemsa's method exactly as we stain our direct films for the detection of the trachoma inclusions. In examining these smears we found that cultures of these bacilli, as they grew older, altered significantly both in their morphology and in their staining reactions to Giemsa. Twenty-four-hour cultures show dense clumps of short bacilloid or coccoid forms taking a comparatively deep

TABLE 6.

Coincidence of Hemoglobinophilic Bacilli and Trachoma Inclusions in Cases Examined Both by Direct Smears and by Cultures.

Diagnosis	Trachoma Inclusions		Hemoglobinophilic Bacilli		Remarks
	+	−	+	−	
"Old trachoma"	9	12	7	14	Seldom any growth on original plates. Growth in "enriching media."
Papillary conjunctivitis	184	53	177	90	In many of the recovering cases bacilli and "inclusions" are found irregularly.
Follicular conjunctivitis	2	22	14	10	
Acute catarrhal* conjunctivitis	17	71	45	43	Among these cases are included 38 cases of gonorrheal ophthalmia, 9 of which showed trachoma inclusions and recovered in from 3 weeks to 2 months. The others were cases of acute catarrh accompanied by hemoglobinophilic bacilli which recovered in average of 6 weeks.
Folliculosis	0	18	0	18	No "carriers" found.
Other conditions	0	12	4	8	Most of these cases were blepharitis and keratitis accompanied usually by slight catarrh. Examined only one in this way.

* A number of cases (20) diagnosed first as acute contagious conjunctivitis developed later papillary conjunctivitis. These are included here among the papillary conjunctivitis cases.

blue stain exactly as do the early granules of the trachoma inclusions (the "initial bodies of Lindner"). The morphology varies somewhat with the age of the inoculated culture, the date from isolation, the kind of medium, and the strain. In 48 hours the forms become somewhat more irregular. Then in 3 days most of the bacilli have become extremely minute, many showing only as reddish granules (the "elementary bodies of Prowazek"), while scattered through the culture are swollen spheroidal bodies taking a fainter clear blue stain (the larger "initial bodies of

Prowazek"), in some of which are minute reddish granules (more of the "elementary bodies of Prowazek"). A number of irregular light blue bodies are also scattered through the culture. Where the bacteria are densely grouped more red granules may appear in the center of the group than at the periphery and more blue bodies at the periphery than in the center. In short, all of the changes described by Prowazek and others as characteristic of trachoma inclusions are seen in the growing cultures of these hemoglobinophilic bacilli (Plate 2, Figs. 1–6, inclusive).

Similar day-to-day studies were undertaken with the other types of bacteria found most frequently in the eyes diagnosed as trachoma, e.g., streptococci, staphylococci, gonococci, a minute gram-negative, non-hemoglobinophilic bacillus not before described, found in a few cases of papillary conjunctivitis, xerosis bacillus, and *Micrococcus tetragenus*, but in none of these varieties except the gonococcus were similar changes found in the same marked degree. Cultures of the tetragenus coccus, isolated repeatedly from a case of chronic conjunctivitis, showed some dense bunches but practically no red granules. This corresponded with what was found in direct smears from the case. Many of the epithelial cells contained the tetrads, some distinct and some in dense clumps, approaching in morphology the early trachoma inclusions found in ophthalmia neonatorum, but no forms containing reddish granules were seen.

The fact that the gonococcus cultures showed such definite appearances similar to the trachoma inclusions led us to make a special study of a series of ophthalmia neonatorum cases, 38 in all. The cultural technic employed was the same as that used for the other cases and we were on the lookout particularly for gonococci and hemoglobinophilic bacilli. In most of these 38 cases, treatment had already been begun when our cultures and films were made; in fact, many of the cases were already near recovery, so we were not able to get a complete cultural and morphologic picture; however, the results as given in Table 7 are rather suggestive. Gonococci were demonstrated by us in both smears and cultures in 10 cases, in smears alone in 9 cases, in cultures alone in 3 cases, and in neither culture nor smear in 16 cases. Most of the cases

giving negative results and some of the others were examined several times. This picture of irregular results is one that might be expected from recovering cases. From only three of these cases were hemoglobinophilic bacilli isolated. In 9 cases definite

TABLE 7.

Coincidence of Trachoma Inclusions, Gonococci, and Hemoglobinophilic Bacilli in Cases of Ophthalmia Neonatorum.

Number of Case	Smears			Cultures		Case Termination (Weeks)
	Trachoma Inclusions	Transition Forms	Gonococci	Gonococci	Hemoglobin-ophilic Bacilli	
1	−	−	−	−	−	4
2	−	−	−	−	−	4
3	−	+	+	−	−	5*
4	+	+	−	+	−	5*
5	−	−	−	−	−	5*
6	+	+	−	+	−	8*
7	−	+	+	−	−	8
9	+	+	+	−	−	3*
M 1	−	−	−	−	−	4
M 2	−	−	−	−	−	2
M 3	+	+	+	−	−	3*
M 4	−	−	−	−	−	2
M 5	−	−	−	−	−	5*
M 6	−	−	−	−	+	6*
M 7	−	−	−	−	−	5*
M 8	−	−	−	−	−	4*
M 9	+	−	−	−	−	5*
M 11	−	−	−	−	−	2
M 12	−	−	+	+	−	4*
M 13	+	−	−	−	−	4*
M 14	−	+	+	−	−	4*
M 16	−	+	+	+	−	7
M 18	−	−	+	−	−	2 (died)
M 19	−	−	−	+	−	3 "
M 20	−	+	+	−	−	4
M 21	−	+	+	+	−	4
B 136	−	−	+	+	−	8
1,682	+	+	+	+	−	7
1,686	−	−	+	+	−	6*
1,687	−	−	−	−	−	2
1,707	−	−	−	−	−	2
1,708	−	−	+	−	+	3
1,709	−	−	+	+	−	3*
1,710	−	−	−	−	−	2
1,714	−	−	+	−	−	8
1,715	−	−	+	+	−	3
1,716	−	+	+	+	−	6
1,720	+	+	+	+	+	4

* Reported well when they left the hospital just before our last visit.

trachoma inclusions were seen, in only one of which hemoglobinophilic bacilli were found as well, while in 7, gonococci were found. In two showing trachoma inclusions no definite organisms were demonstrated in either smear or culture. A minute study of the direct smears from these eyes apparently throws more light on the subject. In all of the inclusion cases where gonococci are

found, apparent transition forms between gonococcus and inclusion are very evident, and we find from a further microscopic study of these slides that the inclusions on the whole present certain characteristics different from those found in our series from school children, produced, according to our hypothesis, by nests of growing hemoglobinophilic bacilli. The inclusions, produced as we think by the gonococci, are characterized (1) in early stages by being more definitely coccus-like at the periphery of the clump and more densely clumped in the center; (2) in the later stages by showing on the whole smaller red granules than those produced by the hemoglobinophilic bacilli. These same differences are observed in a comparative day-to-day study of pure cultures of these two species of bacteria (compare Figs. 7–12 inclusive in Plate 2). We have found that different strains of each species vary in size of granules produced, hence the second characteristic is not a clean-cut one.

From this comparative morphologic study of "inclusions" and cultures we have reached the following conclusions:

1. The well known power of epithelial cells of the conjunctiva as well as epithelial cells of other parts of the body to harbor foreign invaders (e.g., erythrocytes, leukocytes, several species of microorganisms, notably gonococci and hemoglobinophilic bacilli) may be in evidence in any stage of inflammation.

2. Under certain conditions of infection, chief of which are, probably, a lessened virulence on the part of the invading organism and a lessened susceptibility on the part of the invaded cells, or one or the other of these conditions, some invaders (notably gonococci and hemoglobinophilic bacilli) may grow to a varying extent within the cells in groups or nests and thus produce the characteristic changes described as trachoma inclusions.

3. Under these conditions, too, growth of the invader may not be limited to the epithelium alone but may extend to the deeper tissues. According to the extent and kind of irritation produced by such growth, a variable degree of proliferating inflammation of the surrounding tissue may be established, which later, if unchecked, may go on to the formation of fibroid tissue.

If it be true that both gonococci and hemoglobinophilic bacilli

(and possibly other species of bacteria) may grow in epithelial cells in nests presenting a morphologic cycle similar to that described for the trachoma inclusions, it explains, as one of us (Williams) has pointed out, the contradictory findings reported in cases of "inclusion ophthalmia," gonorrheal conjunctivitis, and *inclusion inflammation* in the mucous membranes of urethra and vagina ("trachoma of the urethra or vagina"[?]) and of other parts of the body. That is, in a large number of cases of "papillary conjunctivitis" and a certain number of cases of ophthalmia neonatorum, as well as in a certain number of cases of inflammation of the mucous membranes of other parts of the body (e.g., vagina, urethra), the trachoma inclusions found are due to one or more varieties of hemoglobinophilic bacilli; in a certain number of cases of gonorrheal ophthalmia as well as in gonorrheal inflammation of the mucous membranes of other parts of the body, the trachoma inclusions are due to the gonococcus.

According to one of the later reports of Leber and Prowazek and the recent report of Noguchi and Cohen, certain inclusion conjunctivitis cases may be caused by microorganisms other than the two mentioned above.

OTHER MICROORGANISMS FOUND.

Since several varieties of organisms other than the hemoglobinophilic bacilli and the gonococci are frequently present in cases diagnosed clinically as trachoma, the question of their action may now be discussed. Of course, from practically all cases with any secretion more than one variety of microorganisms may be grown. Particularly is this true when a fluid medium is used for an original culture. Just how far enriching fluid media may be relied upon to aid us in determining the pathogenicity for the host of the organisms isolated by this method has not yet been accurately determined. We know, however, that under certain conditions, organisms exerting an active pathogenic action in the body of the host are difficult to isolate and to grow immediately after isolation on artificial culture media. An enriching medium under these circumstances may be the only means to demonstrate the most harmful organism present. But, without doubt, it cannot give

us a correct idea of the number of organisms present in the lesion at the time the culture was taken. We must have in mind these and other questions already considered when we try to interpret all of the bacteriologic findings in these studies.

Staphylococci.—We see by Table 3 that the *Sta. pyogenes* group was demonstrated in nearly all of the cases. But in only comparatively few of these did a large number of colonies grow on the original plates, and these cases presented no uniform or related clinical picture. Original plates from the great majority of the cases showed few or no staphylococcus colonies. In the latter case, the enriching medium used showed them after 24 hours.

The well known power of the group of *Sta. pyogenes* to vary in pathogenicity, coupled with the effect of the varying degrees of susceptibility or immunity of the host, could produce, as many have pointed out (see Duane and Hastings), many grades of pathogenic action. To determine the exact conditions and limits of pathogenicity of this group would require much more work than we have been able to give in this study. We do know, however, that this group of organisms has been found in a number of cases of ulcer of the cornea, and in several cases of acute follicular conjunctivitis, acute exacerbation of trachoma, and acute pannus as apparently the predominating organism present (judging from growth on original plates) in the curetted conjunctiva.

It seems, then, that in certain cases these cocci may play a most important part, not only in certain definite infections, but also in helping along a case of follicular conjunctivitis to become a "bad case of trachoma."

Streptococci.—We see by reference again to Table 3 that many streptococci were found. These were all of the short-chained, non-hemolyzing type. In order to help determine the relationship of these strains to the great group of pneumo-streptococci, 20 strains isolated respectively from 20 successive cases showing these streptococci in original cultures were studied for both their hemolyzing and their fermentative powers. Table 8 (p. 326) shows the condensed results of this study.

We see by studying this table that 45 per cent of these strains are typical pneumococci, while 55 per cent may be classed as

TABLE 8.*

Fermentation and Hemolysis Tests of Pneumo-Streptococcus Group.

	Culture	Milk			Neutral Red			Saccharose		Lactose		Raffinose		Inulin			Salicin		Mannite		Hemolysis	
		Test	Clot	Acid	Test	Reaction	Gas	Test	Acid	Test	Acid	Test	Acid	Test	Acid	Clot	Test	Acid	Test	Acid	Green Producer	Hemolysis
Pneumococcus	21–79	3	+	+	2	−	−	1	+	1	+	1	+	4	+	−	2	+	2	−	+	±
	H 1,658	3	+	+	2	+	−	1	+	1	+	1	++	4	+	−	3	+	3	−	++	−
	545	2	+	+	1	−	−	1	++	1	++	1	+	2	±	−	3	?	3	−?	+	−
	J 38	2	+	+	1	+	−	1	++	1	++	1	++	4	+	−	2	+	3	+	++	−
	150(?)	2	−	+	1	+	−	1	++	1	++	1	+	3	?	−	2	+	3	−?	++	−
	PS 371	2	+	+	1	+	−	1	+	1	++	1	++	3	±	−	2	+	2	−	++	−
	H 2,225	2	+	+	1	−	−	1	±	1	++	1	++	3	+	−	2	±	2	−	++	−
	PS 108	2	+	+	1	−	−	1	++	1	+	1	++	2	±	−	2	+	2	−?	++	−
	21–394	1	+	+	1	+	−	1	++	1	++	1	++	1	±	−	1	++	1	−?	+	−
Type A	Res 375	3	+	+	2	+	−	1	+	1	+	1	±	3	−	−	3	−?	3	−	++	−
	2,185 A	2	±	+	1	+	−	1	±	1	+	1	±	4	−	−	2	−	3	−	+±	−
	562	2	+	+	1	+	−	1	++	1	++	1	−	4	−	−	2	−	3	−	+±	−
	1,035	1	+	+	1	++	−	1	++	1	++	1	++	1	−	−	1	−	1	−	+	−
Type C	M 19 Re	3	+?	+	2	−	−	1	+	1	+	2	?	4	−	−	3	−?	3	−	+	−
	G 520	2	+	+	1	−	−	1	+	1	++	1	−	3	−	−	2	?	3	−?	+	−
	H 2,224	2	−	+	1	−	−	1	+	1	+	2	−	2	−	−	2	?	2	−?	++	−
Unclassified	311 A	2	+	+	1	+	−	1	±	1	++	1	±	2	−	−	2	++	2	−?	+	−
	21–290	3	±	+	2	+	−	1	+	1	+	1	+	4	−	−	3	+	3	−	+	−
	2,253	1	+	+	1	+	−	1	+	1	+	1	+	1	−	−	1	++	1	−	+	−
	G 174	1	−	+	1	+	−	1	+	1	+	1	−	1	−	−	1	+	1	±	−	±

*Elser's technic was followed in making the sugar media. Observations were made up to two weeks. After one week no further change was seen. ++=Very marked acidity; +, marked acidity; ±, moderate acidity; ±, slight acidity; ±, trace of acidity.

Str. viridans (producing in blood-agar plates a green halo about each colony instead of a zone of hemolysis), or if Gordon's classification is used, we could have the latter divided into *Str. pyogenes*, 15 per cent, *Str. salivarius*, 20 per cent, and unclassed streptococci, 20 per cent.

The fact that so many of these streptococci are of the type that is supposed to produce chronic inflammations in other parts of the body (e.g., in chronic articular rheumatism, endocarditis, etc.) is very significant. They seem to be more frequent in the subacute and chronic cases. In the pannus cases studied where they are very frequent, the part played by these streptococci may be of great importance. This point is still under study. We must remember that members of this group of organisms show also a variable pathogenic power, and that the host responds with a variable degree of susceptibility or immunity.

The other species of microorganisms noted in Table 3 were found too infrequently to be considered as more than incidental aids in presenting an individual picture of a stage in chronic conjunctivitis which might be considered clinically as trachoma in exacerbation. Thus there were several of these cases, notably the tetragenus and the yeast infections, that passed through a clinical course of conjunctivitis lasting several months, and were diagnosed for some time as papillary trachoma with follicles. These eyes finally became completely normal. They were all through most of their course mixed infections; one of the yeast cases had also the hemoglobinophilic bacilli, the other two yeast infections and the two tetragenus infections showed streptococci as well.

Anaerobic cultures.—When the cultural work began deep tubes of the different media used were inoculated and grown under albolene. We discovered the interesting fact that under these conditions both hemoglobinophilic bacilli and gonococci would grow in the depths of the tubes. When we found that no unidentified organisms grew in this way in a series of cases diagnosed then as trachoma and which contained trachoma inclusions, this method of growth was discontinued. That we may have missed important organisms by discontinuing this method is possible. We have

not worked out all of the organisms found in the tubes of enriching media. We have diagnosed them from their morphology and staining characteristics as belonging to one or the other of the groups mentioned in Table 3. In a few cases of papillary conjunctivitis we have found a minute gram-negative bacillus which grew abundantly on all media. In a few other cases in the same condition, we found a minute gram-amphiole bacillus which we did not isolate. No doubt a number of cocci escaped us but none of these were frequent enough, either in the original plates or in the enriching media, to be considered of pathogenic importance.

In summing up this cultural work, we wish to emphasize the following points:

1. Hemoglobinophilic bacilli are present much more frequently than was thought in cases of chronic as well as of acute conjunctivitis.

2. These bacilli were obtained in abundant cultures from all cases examined that were diagnosed clinically as "pink eye" or acute contagious conjunctivitis. No classic Koch-Weeks bacillus was found; therefore the bacillus apparently producing "acute contagious conjunctivitis" in these cases is a strict hemoglobinophile.

3. None of the 424 strains of hemoglobinophilic bacilli isolated in this series can so far be differentiated from the influenza bacillus.

4. These bacilli are found in cases of papillary conjunctivitis ("acute trachoma") coincidently with trachoma (Prowazek) inclusions.

5. These bacilli in pure cultures present morphologically the same cycle of development as that shown by the trachoma inclusions seen in direct smears from cases of papillary conjunctivitis, and these direct smears also show transition forms between bacilli and inclusions. Therefore bacilli and inclusions are probably identical in these cases.

6. Among all of the species of microorganisms isolated from cases of conjunctivitis only the gonococcus shows in pure cultures a cycle of development similar to that of the hemoglobinophilic bacilli; but the fact that the former presents certain characteristics corresponding to the characteristics found in the trachoma

inclusions seen in direct smears from the host cases (gonorrheal ophthalmia neonatorum), coupled with the fact that transition forms between gonococci and inclusions are also seen in these smears, makes it probable that inclusions and gonococci are identical in *these cases*.

7. Trachoma inclusions, then, are simply intracellular nests of growing bacteria.

8. Among the other microorganisms found in these cases certain staphylococci and certain of the non-hemolyzing streptococci may play an important part in the chronicity of certain cases, especially in cases of pannus.

VI. DISCUSSION, SUMMARY, AND CONCLUSIONS.

Anna Wessels Williams.

DISCUSSION.

The chief practical questions this study calls forth are: (1) Does a specific disease called trachoma exist in New York City? (2) If it does exist, how many individuals does it affect? (3) How contagious is it?

While our study has corroborated the opinion of others that there is no clear-cut clinical entity called trachoma, it has not ruled out the possibility of there being a specific disease answering in part at least to the classic descriptions of trachoma.

In considering the question of the present existence of such a disease in New York City, we must bear in mind several facts: (1) Since 1897 no case diagnosed as "active trachoma" has been allowed to enter New York City through its port. Without doubt this has lessened in a marked degree fresh foci of eye infections. (2) The poor in New York City, among whom most of the cases diagnosed as trachoma occur, live on the whole more hygienically than they do in their native countries where trachoma is said to be frequent. (3) The methods of caring for the eyes of the citizens of New York City are becoming better year by year, through the employment of various agencies, such as school inspections and hygienic procedures, eye clinics, home visits, public lectures, and

so on. The resulting conditions are directly preventive of the development of an infection from any case of "beginning trachoma" which either may have been infected from an "old case" or a "carrier" or may have slipped through the port, as it might readily do even with the port's strict oversight if the majority of cases begin insidiously, as most authors think.

Under these conditions, therefore, we may still be having many new cases of trachoma which either abort or run a comparatively mild atypical course to cure.

Our studies so far show either that this conclusion is correct or that at present we have practically no cases of trachoma among the public-school children on the lower East Side of New York City in a district supposed to be a hotbed of trachoma.

For among these 4,000 or more cases of conjunctival affections in the school children under observation for an average of 3 years and seen from or near the beginning of the disease, none have so far shown cicatricial formation except those among the few operated upon (expression) who show "operative scars." Moreover, the great majority of all of the cases, after passing through a comparatively short chronic course, have presented and still present apparently normal conjunctivas.

That our cases have been under exceptional hygienic and medical treatment from the beginning of the diseased conditions may account in great measure for this result. But whether they are cured light cases of trachoma, or simply quiescent cases of trachoma ready to show specific exacerbation under certain conditions; or whether they are cases which were infected only with germs other than a specific trachoma germ leaving their conjunctivas subject to reinfection and ready to progress, if later they become neglected, to "non-trachomatous pannus or cicatricial formation" or both, may be answered only by future study.

Among this large group of cases only eight during our period of observation have developed a condition diagnosed by ophthalmologists as pannus. Whether this condition in these cases is a specific manifestation of a trachoma virus, we cannot say. All of these cases but one have now practically clear corneas and normal conjunctivas. All of them had, antedating and accompanying

the pannus, more or less papillary conjunctivitis and follicle formation. From all of the eight curetted conjunctivas members of the pneumo-streptococcus group were obtained. A polyvalent vaccine made from strains of the pneumo-streptococcus group seemed to have a quickening effect upon the march of these cases to recovery. Whether or not this was simply coincidence it is too soon to say.

Among our cases over 300 showed a sluggish papillary thickening of the conjunctiva accompanied in most cases by mucopurulent secretion and by marked follicle formation apparently through the depths of the conjunctiva. The occurrence in these cases of trachoma inclusions and hemoglobinophilic bacilli at the height of the affection and irregularly throughout the course, coupled with the morphologic similarity of bacilli and inclusions (see Plate 2), has led us to the hypothesis that hemoglobinophilic bacilli growing in nests in the epithelial cells of the conjunctiva are identical with trachoma inclusions in these cases and are the cause of these subacute and chronic cases (papillary conjunctivitis with or without follicles) as well as of acute conjunctivitis. Whether or not this hypothesis is correct, the results from its practical application—i. e., treating more minutely and following up all of our acute cases of conjunctivitis until we are sure of cure, as well as hunting up and treating acute cases in the families—give evidence that it works.

The fact that we found trachoma inclusions in 8 cases of acute catarrhal conjunctivitis, cured in an average of 8 weeks, while transition stages between bacilli and inclusions in these and other acute cases were frequently seen, lends more evidence to this hypothesis.

There is no question, from our studies, but that bacteria other than hemoglobinophilic bacilli may grow in nests in the epithelial cells of the conjunctiva and so produce appearances like trachoma inclusions, e.g., gonococci in cases of gonorrheal ophthalmia; and it is possible that we have missed a "specific trachoma bacterium" in some of our cases. But the evidence seems strongly in favor of the truth of our hypothesis at least as regards the great majority of the cases of conjunctivitis in our public school children.

Furthermore the evidence admits the possibility that neglected cases, or especially susceptible cases, infected with hemoglobinophilic bacilli may result in a fibrous tissue stage.

As regards the question of the degree of infectivity of these cases, again it is difficult to give a clear-cut answer, owing to the variableness of the clinical picture.

On the whole we have found that our schools at present seem to play an immaterial part in the spread of the chronic eye affections. Among more than 100,000 school children in the 60 schools we have studied more closely on the lower East Side, only about 3,000 have shown during the past 3 years more or less chronic affections of the conjunctiva and over 2,000 of these have been only mild folliculosis. As for the more serious cases, no school stands out particularly as a focus of infection. The few schools that have the most cases have children coming from the most unhygienic families. In isolated instances in the past, infections apparently contracted in the classroom have been reported, but none have come under our observation during the past 4 years. The possibility of the spread of eye infections in schools may be reduced to a minimum by the employment of proper hygienic precautions such as have been carried out in Public School 21, described in Dr. von Sholly's paper of this series.

In regard to home infection we have made estimates from two groups of cases. The first estimate (given in Miss Kurtz's paper) is made from the average chronic cases of papillary conjunctivitis. The percentage of probable infection was found to be 22.

In our other estimate made later, in which were included a number of the "old trachoma" cases, as well as cases of papillary conjunctivitis, Miss Gurley found that in 87 families with an average of 7.5 individuals to the family (653 individuals), 144 individuals had a condition diagnosed by most ophthalmologists as trachoma. Of these, 57, excluding the one in each family presumably infected from the outside, developed during the period of infection a chronic papillary conjunctivitis with follicles, that is, about 10 per cent of the 566 individuals exposed, a smaller proportion than that usually given. In the families of the 38 pannus cases we have had (most of them among the chronic adult cases)

a remarkably low percentage of cases of chronic conjunctivitis developed in the exposed individuals.

That our campaign in behalf of personal hygiene, both in home and in school, carried on during the whole course of these studies helped lessen the spread of infection from these cases, especially in the families, we think probable, not only from observations of isolated cases, but because at present so few subacute and chronic cases seem to be developing in our lower East Side district, while they are still reported as prevalent in other crowded sections of the city.

Unless we are able to exercise strict supervision over the cases of acute conjunctivitis occurring in schools and homes, and to carry out the school and home instructions in personal hygiene, we are convinced that after a time we may have large numbers of well-developed chronic cases, a certain percentage of which may finally show pannus and cicatricial changes. In short, they may present the picture seen at present in the neglected cases among the Kentucky mountains and on some of our Indian reservations.

SUMMARY.

1. The written descriptions of trachoma do not agree as to a clinical or a pathologic entity.

2. Among the children of 60 public schools in the crowded lower East Side of New York City (supposed to be a hotbed of trachoma) during the past 4 years we have found no cases of conjunctival affections answering in their entirety to the classic descriptions of trachoma.

3. None of the 3,000 and more cases of *follicular* affections in these children have developed cicatricial changes due to infection; on the contrary the great majority of them now present normal conjunctivas. We attribute these results chiefly to the following measures.

a) The carrying out of preventive methods of treatment such as ophthalmia schools and school clinics, summer camps, mothers' demonstration classes, home instructions, and "follow-up" work.

b) More minute care of acute, as well as of chronic cases, including the specific treatment given chiefly at clinics and the follow-up work in the homes.

c) Non-operative procedures in a large number of cases formerly operated upon.

4. Hemoglobinophilic bacilli are found in a large proportion of the cases of chronic conjunctivitis (especially of the papillary type) as well as in those of acute catarrhal conjunctivitis (including clinically typical "pink eye").

5. These bacilli, tho they probably represent several different varieties, have not yet been differentiated from *B. influenzae*.

6. These bacilli are found coincidently with trachoma inclusions in a high percentage of the cases of subacute and chronic conjunctivitis when examined from the beginning.

7. These bacilli show morphologic and staining characteristics similar to those seen in trachoma inclusions; and transition forms between bacilli and inclusions are frequently seen in these cases.

8. In a few cases of acute catarrhal conjunctivitis, trachoma inclusions and transition forms have been found coincidently with hemoglobinophilic bacilli and in several other acute cases transition forms have been often found.

9. Many of the cases of chronic papillary conjunctivitis not actually followed from the beginning by us give a history of repeated acute attacks, and several cases beginning with us as acute contagious conjunctivitis passed into the chronic condition called papillary conjunctivitis.

10. Cell inclusions similar to the trachoma inclusions found in the papillary conjunctivitis cases are found in cases of gonorrheal ophthalmia neonatorum, cultures from which show only gonococci. These inclusions differ somewhat from those found in the papillary conjunctivitis cases, and transition forms between gonococci and inclusions are seen in smears from the former cases.

11. Members of the pneumo-streptococcus group are frequently found in chronic as well as in acute cases, especially in those cases showing pannus.

CONCLUSIONS.

1. Trachoma inclusions are nests of growing bacteria in epithelial cells—hemoglobinophilic bacilli in certain cases of papillary conjunctivitis, gonococci in certain cases of gonorrheal conjunc-

tivitis and possibly other bacteria in certain other cases of conjunctivitis.

2. Under minute hygienic and medicinal treatment, such as is outlined in some of the preceding articles, the great majority, if not all, of the cases of conjunctival affections of children may run a benign course resulting in normal conjunctivas.

3. Comparatively few, if any, cases of chronic conjunctivitis develop in individuals exposed, if the rules of general and personal hygiene are carried out.

4. If trachoma is present or should be introduced among our school children it may be controlled by methods which are within practical limits.

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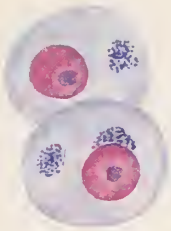
PLATE 2.



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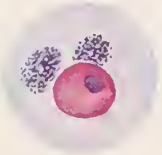

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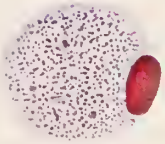

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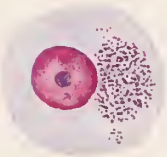

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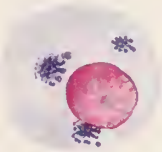

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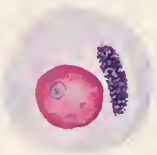

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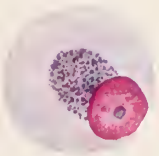

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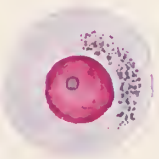

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EXPLANATION OF PLATE 2.[1]

Figs. 1, 2, 4, and 5.—Epithelial cells containing trachoma inclusions in films taken from conjunctiva in case of papillary conjunctivitis (formerly diagnosed acute trachoma).

Figs. 3 and 6.—Pure cultures of hemoglobinophilic bacilli from same case.

Fig. 1.—Initial forms of inclusions staining deep blue, some showing as small bacilli.

Fig. 2.—Later stage of inclusion showing beginning appearance of reddish granules.

Fig. 3.—Twenty-four-hour culture on blood agar of hemoglobinophilic bacilli showing coccoid forms similar in size and staining to those seen in early trachoma inclusions.

Fig. 4.—Later trachoma inclusions almost filling epithelial cell showing many reddish granules and irregularly shaped light blue bodies.

Fig. 5.—Another later stage showing beginning absorption (?) of the inclusions.

Fig. 6.—Four-day culture of the bacillus in Fig. 3, showing many reddish granules and irregularly shaped light blue masses similar in size and staining to those seen in later trachoma inclusions.

Figs. 7, 8, 10, and 11.—Epithelial cells containing trachoma inclusions in film taken from conjunctiva in case of gonorrheal ophthalmia neonatorum.

Figs. 9 and 12.—Pure cultures of gonococci from same case.

Fig. 7.—Early stage of inclusions showing morphologically typical cocci still at edge of inclusion.

Fig. 8.—Later stage of inclusion showing dense group and beginning minute reddish granules.

Fig. 9.—Twenty-four-hour cultures of gonococci on blood agar, showing staining and groups similar to those seen in epithelial cells in "early stage."

Fig. 10.—Later stage of inclusions from gonorrheal case, showing minute reddish granules.

Fig. 11.—Still later stage of inclusion showing irregular bluish bodies and scattered minute reddish granules.

Fig. 12.—Three-day pure culture of gonococci showing minute reddish granules and swollen bluish bodies similar to those seen in late inclusions.

[1] All preparations stained by Giemsa and magnified about 1,200 diameters.

INFLUENCE OF PROTEIN CONTENT ON THE ABSORP TION OF ANTITOXIN AND AGGLUTININ INJECTED SUBCUTANEOUSLY.*

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(From the Research Laboratory, Department of Health, New York City.)

The subject of this communication has become important because of the fact that diphtheria and tetanus antitoxins are supplied in this country most commonly in the form of the refined and concentrated globulin preparation. The methods employed in the United States are those developed in this laboratory by Atkinson, Gibson and Banzhaf. These antitoxic preparations contain that fraction of the serum protein designated pseudoglobulin. In the actively immunized horse, the antitoxins are intimately associated with the pseudoglobulin, and by the process of "salting out" may be separated with it from the other proteins, such as albumin, euglobulin, etc. By these methods, a concentrated preparation of diphtheria antitoxin, for instance, may be prepared containing 2,000–3,000 units per cubic centimeter with a protein (pseudoglobulin) concentration of 18–20 per cent, starting with an original serum containing 400–600 units and a protein content of 8–9 per cent. This concentration of antitoxin is obtained about equally by the elimination of the non-antitoxic proteins and by the abstraction of water. The removal of the water is merely a convenience, while the removal of the proteins, together with the changes produced by the heating employed in the process, removes certain deleterious substances which are present in the whole serum. Careful observation of cases in the hospital wards under our supervision has shown that serum reactions are less frequent in patients receiving the refined antitoxin than in those who have had the whole serum product.

In the treatment of diphtheria, it is a well recognized fact that the antitoxin should reach the general circulation as quickly as possible after its administration. The injection of the antitoxin

* Received for publication January 15, 1914.

directly into the blood stream is the only way that fully meets such requirements, but owing to the technical difficulties encountered, this method is not commonly employed by the practitioner. Instead, the subcutaneous injection is the means of administration in general use. In connection with the subcutaneous method of administration, this question has arisen: Does the highly concentrated antitoxic preparation, with its increased protein content, pass as rapidly from the subcutaneous or muscular tissues to the blood as do preparations having a larger volume of fluid of lower protein and lower antitoxin concentration? This question becomes of special interest in connection with certain commercial preparations of concentrated antitoxin containing a percentage of protein sometimes reaching $3\frac{1}{2}$ times that of the native antitoxic serum.

The influence on absorption of the protein concentration in the antitoxin solutions was considered by us when we began to use the refined antitoxin. The results of our investigations convinced us that a concentration to at least double that of an immunized horse serum had little, if any, appreciable effect in retarding absorption. The results obtained by L. E. Walbum[1] with the absorption of agglutinin in rabbits which showed that, in those animals in which the protein concentration of the injected fluid was equal to that of the blood serum, the agglutinin was more rapidly absorbed than from solutions containing either a higher or lower protein concentration caused us to attempt a new investigation of the subject.

Walbum states at the beginning of his paper that on account of work carried out by Henderson-Smith and E. Tobin he assumed that the conditions affecting the absorption of antitoxins were similar to those affecting the absorption of agglutinins. He went even farther and assumed that the conditions affecting the absorption in the subcutaneous tissues of rabbits of *B. coli* agglutinin produced by the goat and contained in goat serum or goat globulins were similar in the case of the absorption of horse-produced antitoxin from the tissues of man. In somewhat condensed form, his conclusions are as follows: The absorption of agglutinin from the subcutaneous tissues is much slower from serum containing 15 per cent of protein than from that containing the normal amount (6.2 per cent, goat). When the more concentrated protein solution is diluted with normal saline solution, so as to be reduced to the same protein content as the normal, the agglutinin is absorbed equally from both solutions. Less than the normal concentration retards the absorption from subcutaneous tissues, but increases the rate of absorption from that injected into the muscles. The absorption from the peritoneal cavity is not affected by the protein concentration of the serum.

A critical examination of Walbum's results gives the impression that the difference in rapidity of absorption of the agglutinin from the solutions of different protein concentrations was less marked than Walbum judged. Thus, if the rabbit receiving the concentrated protein in his Experiment 1 is compared with the rabbit receiving the normal protein percentage in the Experiment 7, the absorption curves are practically identical, if allowance is made for the different weights of the animals. The

[1] *Ztschr. f. Immunitätsf.*, 1912, 12, p. 546.

similarity of the rate and degree of absorption of the agglutinin from solutions of different protein concentrations is strikingly shown in Walbum's Experiment 10. We reproduce his Table 1, simply adding figures in brackets correcting the amounts to correspond with a weight of 1,950 grams.

The figures in this table show that in this experiment the increased protein concentration had no appreciable effect in retarding the rate of absorption or the amount. While the lessened protein concentration appeared to decrease the rate and amount of absorption after the first day, yet it apparently increased both in the earlier and more important period.

TABLE 1.

VERSUCH X. SUBKUTANE INJEKTION (WALBUM).

	14,95 PROZ. EIWEISS KANINCHEN, 1,950 G.		10 PROZ. EIWEISS KANINCHEN, 1,950 G.		7 PROZ. EIWEISS KANINCHEN, 1,700 G.		4 PROZ. EIWEISS KANINCHEN, 1,700 G.		1 PROZ. EIWEISS KANINCHEN, 1,750 G.	
		Aggl.-Einh. in 1 c.c. Ser.		Aggl.-Einh. in 1 c.c. Ser.		Aggl.-Einh. in 1 c.c. Ser.		Aggl.-Einh. in 1 c.c. Ser.		Aggl.-Einh. in 1 c.c. Ser.
Vor der Injektion....	>0,2		>0,2		>0,2		>0,2		>0,2	
16 Std. nach der Injekt.	0,03	33,3	0,0225	44,4	0,0185	54 [47]	0,015	67 [58.4]	0,013	77 [69.1]
26 Std. nach der Injekt.	0,007	143	0,007	143	0,007	143 [124.6]	0,005	200 [174.3]	0,007	143 [128.3]
40 Std. nach der Injekt.	0,0035	286	0,003	333	0,0029	340 [296.4]	0,004	250 [217.9]	0,0055	182 [163.3]
50 Std. nach der Injekt.	0,0029	345	0,00275	364	0,0025	400 [348.7]	0,0037	270 [235.3]	0,004	250 [224.3]
64 Std. nach der Injekt.	0,0025	400	0,00225	444	0,0021	476 [414.9]	0,0035	286 [249.3]	0,0037	270 [242.3
74 Std. nach der Injekt.	0,0026	384	0,0022	455	0,002	500 [435.8]	0,0035	286 [249.3]	0,0037	270 [242.3
88 Std. nach der Injekt.	0,00275	364	0,00225	444	0,0021	476 [414.9]	0,0037	270 [235.3]	0,004	250 [224.3]
98 Std. nach der Injekt.	0,004	250	0,0025	400	0,0024	417 [363.5]	0,005	200 [174.3]	0,0049	204 [183]
136 Std. nach der Injekt.	.....	.....	0,004	250	0,0035	286 [249.3]	.....	.....	.....	.....

ORIGINAL INVESTIGATIONS.

As it is possible that the sera of different animals vary in their absorption characteristics, we determined to test the effect of protein concentration upon absorption by using the serum of the horse because this animal is almost universally employed to furnish protective sera for man. Because of the importance of this question and the lack of danger of the experiment, we also tested the matter in man as well as in animals.

Our first experiments to test the question of absorption of antitoxin in different protein concentrations were performed upon goats. Nine animals, which were divided into two main groups,

were selected for the tests. Serum samples taken for controls before beginning the experiments showed no appreciable amounts of antitoxin. Each animal received 10,000 units of diphtheria antitoxin by subcutaneous injection in the lower abdominal region. The antitoxin given was incorporated with widely different concentrations of protein (pseudoglobulin), and sufficient NaCl was added to make isotonic (0.8 per cent). Different volumes of the mixtures also were used in the injections. Two animals received a volume of 2.7 c.c. containing 30 per cent solids; 3 animals, 10 c.c. volume, 30 per cent solids; while one received 30 c.c. volume, 30 per cent solids. Three animals received solutions having only 7.5

TABLE 2.

INFLUENCE OF PROTEIN CONCENTRATION UPON ABSORPTION OF ANTITOXIN. GOATS (SUBCUTANEOUS INJECTION).

	10,000 UNITS DIPHTHERIA ANTITOXIN								
	Solids, 30 Per Cent (Protein, 29.2 Per Cent+NaCl, 0.8 Per Cent)						Solids, 7.5 Per Cent (Proteins, 6.7 Per Cent + NaCl, 0.8 Per Cent)		
Number	1 H.	2 H.	3 H.	4 H.	5 H.	6 H.	1 L.	2 L.	3 L.
Volume injected	30 c.c.	10 c.c.	10 c.c.	10 c.c.	2.7 c.c.	2.7 c.c.	10 c.c.	10 c.c.	10 c.c.
Places injected..	3	3	1	1	1	1	3	1	1
After injection	Units	Units	Units	Units	Units	Units	Units	Units	Units
18 hrs.....	7.82	7.86	6.8	5.7	8.37	5.58	8.22	8.75	7.68
36 "	10.2	9.07	8.83	7.6	8.64	6.97	10.72	9.0	8.4
48 "	10.2	8.47	9.12	7.6	8.91	7.16	10.01	8.5	7.92
72 "	8.84	8.47	9.12	7.6	8.64	6.51	9.65	8.0	7.2
96 "	6.8	6.05	7.41	5.7	7.56	6.51	7.86	6.0	7.2

The antitoxic unit strength was first estimated between points of 0.25 units difference. In correcting the figures to a uniform goat weight (50 lbs.) the figures show apparently more accurate estimation.

per cent total solids. As mentioned above, each mixture contained 10,000 units of diphtheria antitoxin, and in 6 of the cases the animals were injected in one place only. While in the remaining 3 instead of injecting in one place, the dose was equally distributed in 3 places in each animal. Blood samples taken from each animal at intervals after the injection had the serum removed by clotting out, and the antitoxic value of each was determined for the following periods: 18, 36, 48, 72, and 96 hours. As a basis for comparison the antitoxic value in units per cubic centimeter was computed relative to an animal weighing 50 lbs. and the results tabulated as shown in Table 2.

The antitoxic unit strength was first estimated between points of 0.25 unit difference. In correcting the figures to a uniform goat weight (50 lbs.) the points of estimation necessarily show different intervals. The weights of the animals were as follows, given in order from left to right: 68, 60.5, 57, 47.5, 54, 46.5, 71.5, 50, and 48 lbs., respectively.

Owing to individual irregularities in absorption, the average values were taken for all the animals which received antitoxin with low solid content, and for those which received antitoxin with high

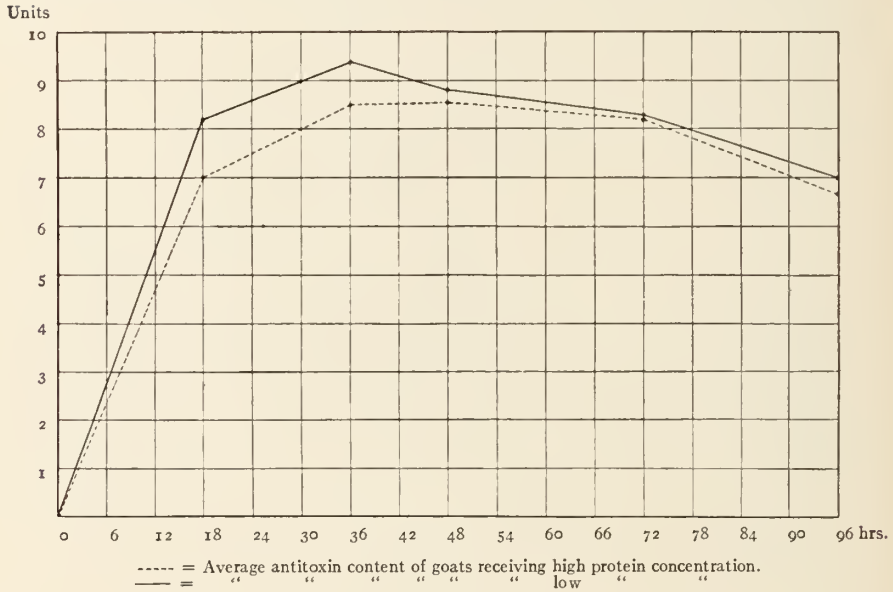

CHART 1.—Comparative absorption from high and low protein concentrations.

solids, and comparisons were made between the two series (Chart 1). It was found that the degree of absorption was somewhat greater for the low solid content mixtures throughout, especially the first period (18 hrs.), in which it was approximately 15 per cent higher; this difference gradually diminished so that at the thirty-sixth hour period, it was less than 5.5 per cent, and at 48 hours, 2.7 per cent.

A second series of tests were carried out upon normal men. Two men received subcutaneous injections of 4.5 c.c. diphtheria antitoxin containing 10,000 units and 18 per cent of total solids. Two

men were given the same dose of antitoxin (10,000 units) to which an equal volume of physiological salt solution had been added, thus doubling the volume (9 c.c.) and reducing its protein percentage one-half. The dose was injected subcutaneously in one place. Bleedings were taken at definite intervals after the injection. The antitoxin content was determined for each blood sample, then the unit value per cubic centimeter was computed to a standard weight (100 lbs.) for comparative purposes. Slight amounts of normal antitoxin were subtracted, when present, for purposes of tabulation. These results are shown in Table 3.

TABLE 3.

MEN (SUBCUTANEOUS INJECTIONS).

INJECTED IN ONE PLACE	10,000 UNITS DIPHTHERIA ANTITOXIN			
	Solids, 18 Per Cent (Protein, 17.2 Per Cent+NaCl, 0.8 Per Cent)		Solids, 9.4 Per Cent (Protein, 8.6 Per Cent+NaCl, 0.8 Per Cent)	
Subject.....	R.	S.	J.	F.
Volume injected....	4.5 c.c.	4.5 c.c.	9 c.c.	9 c.c.
After injection	Units	Units	Units	Units
3 hrs.....	0.45	0.52−	0.12+	0.27+
6 "	0.72	0.91	0.25	0.55
9 "	1.17	1.04	0.31	0.82
24 "	2.70	3.38	0.93	1.86
48 "	3.15	4.03	2.0	2.76
96 "	3.15	4.29	2.0+	2.76−

The unit value of each serum sample in this table has been computed to a standard weight of 100 lbs. for purpose of direct comparison. Weights of men, reading from left to right, are 180, 260, 125, and 138 lbs., respectively.

In these experiments it was found that when the weight was considered the degree of absorption was greater in the men who received the concentrated preparation. This is the opposite of the findings in the tests on goats. Considerable variation of weight existed between the different men, the extremes being 125 lbs. and 260 lbs. Believing that the weight factor and other individual characteristics influenced the foregoing result, it was decided to reinject two of the men. Two of the subjects, one large and one small, were selected from the group and used for reinjection. About 9 weeks after their first injection each one received a second subcutaneous injection (in one place) of 10,000 units of diphtheria antitoxin from the same lot as used in the first series of tests. But,

in this case the smaller man instead of the larger received the 4.5 c.c. volume of 18 per cent total solids preparation. Blood samples were taken from each and the antitoxic content of the serum was determined.

Upon comparison, it was found that the absorption of antitoxin in each man corresponded very closely to the results shown in the first test (Table 4). Therefore, it appears that in this series of tests, the rate and degree of absorption were largely independent of the percentage of protein present within the limits used, but were individual characteristics depending upon the subject receiving the injection. The persistence of the antitoxin in the blood is not affected by the protein concentration.

TABLE 4.

MEN (SUBCUTANEOUS INJECTIONS).

INJECTED IN ONE PLACE	10,000 UNITS OF DIPHTHERIA ANTITOXIN			
Subject	F.		S.	
Volume injected	Second Injection 4.5 c.c.	First Injection 9.0 c.c.	First Injection 4.5 c.c.	Second Injection 9.0 c.c.
Concentration	Solids, 18 Per Cent (Protein, 17.2 Per Cent+NaCl 0.8 Per Cent)	Solids, 9.4 Per Cent (Protein, 8.6 Per Cent+NaCl 0.8 Per Cent)	Solids, 18 Per Cent (Protein, 17.2 Cer Pent+NaCl 0.8 Per Cent)	Solids, 9.4 Per Cent (Protein, 8.6 Per Cent+NaCl 0.8 Per Cent)
After Injection: (Hrs.)	Units	Units	Units	Units
24	1.86	1.86	3.38	3.12
48	2.96	2.76	4.03	3.64
72	2.82	.....	.....	3.9
96	2.82	2.76	4.29	3.9

High and low protein concentrations in the same individuals.

After completing the series of experiments upon man and goats, we decided to perform similar experiments upon rabbits, using a specific agglutinin, thus taking a type of antibody and species for test animal which would allow us to compare our results with those of Walbum. A specific agglutinin for *B. typhosus*, derived from an immunized horse, was fractionated and the pseudoglobulin separated with a portion of the agglutinin; this, after dialysis, was further concentrated in a desiccator over H_2SO_4 at 36–37° C. The concentrated product contained 20 per cent solids, including 0.8 per cent NaCl, and had double the agglutinating value of the

original agglutinating serum. The original agglutinating serum contained 9.88 per cent total solids. Three groups of 3 animals each were given subcutaneous injections in one place (over the abdomen) as follows: (1) 16 c.c. original agglutinating serum (low solids); (2) 8 c.c. volume, concentrated agglutinin preparation (high solids); (3) 16 c.c. volume, composed of 8 c.c. concentrated agglutinin preparation, 8 c.c. normal pseudoglobulin containing the same percentage of total solids. Bleedings were made at intervals

TABLE 5.

RABBITS (SUBCUTANEOUS INJECTIONS)

TYPHOID AGGLUTININS 80,000 UNITS	GROUP 1			AVERAGE	GROUP 2			AVERAGE	GROUP 3			AVERAGE
Weight of Animals	1,560 gm.	1,570 gm.	1,500 gm.	1,543 gm.	1,550 gm.	1,570 gm.	1,510 gm.	1,543 gm.	1,530 gm.	1,600 gm.	1,485 gm.	1,538 gm.
Agglutinin Preparation	Low Solids	Low Solids	Low Solids		High Solids	High Solids	High Solids		High Solids	High Solids	High Solids	
Volume	16 c.c.	16 c.c.	16 c.c.		8 c.c.	8 c.c.	8 c.c.		16 c.c.	16 c.c.	16 c.c.	
Local Reaction	Very Slight	Very Slight	Very Slight		Marked	Moderate	Marked		Marked	Intense	Intense (Abscess)	
After Injection: (Hrs.)	Aggl. U.	Aggl. U.	Aggl. U.		Aggl. U.	Aggl. U.	Aggl. U.		Aggl. U.	Aggl. U.	Aggl. U.	
6	60	70	60	63	40	60	40	46	30	40	10	26
12	120	145	120	128	80	135	100	105	60	90	40	63
24	200	245	250	231	150	235	205	196	150	170	90	136
36	250	345	350	315	200	335	280	271	250	240	125	205
48	300	420	400	373	275	385	330	330	300	315	150	255
60	350	470	450	423	300	415	380	365	350	340	175	288
75	400	520	400	440	350	485	380	405	400	365	200	321
100	325	470	350	381	325	460	330	371	350	390	225	321

The average weight of the rabbits comprising each of the 3 groups being practically identical, the measure of the agglutinating strength of the serum taken from the individual rabbits has not been corrected for a common weight. Instead the average strength of each group has been added.

after injection, and the usual quantitative tests for agglutinins were made upon the clotted-out serum.

In those animals which received the concentrated preparation, a local reaction occurred at the point of injection, followed by infiltration, and, in several animals, a necrosis of the surrounding tissues. As is evident, this reaction greatly disturbed the normal absorptive function. However, it may be of interest to note that when the results of the tests were averaged for each of the 3 groups (Table 5) and curves plotted, they roughly paralleled each other, each group simply showing different degrees of absorption. The

first group of animals which received the original agglutinating serum gave only a slight local reaction and showed the highest degree of absorption of the specific agglutinin. The second group which received 8 c.c. of the concentrated preparation showed less absorption throughout, while the third group with the 16 c.c. volume high solids was still lower. These results would indicate that an infiltration about the injected area must also be an important factor in retarding the absorption of antibodies. The latest period at which the tests were taken revealed that the different sera had approached each other in agglutinating strength. There was slight bacterial contamination of the injected sera. It is not thought that this influenced the local reaction, but it is possible that it did.

CONCLUSION.

The degree of protein concentration which is usually employed to produce the refined and concentrated diphtheria antitoxic globulin preparations has little or no effect in retarding the absorption of the antitoxin from the subcutaneous tissues. The removal of water, if not pushed too far, is therefore a justifiable means of lessening the quantity of fluid to be injected.

Any preparation which causes local inflammatory reaction lessens the rate of antitoxin absorption.

SERUM SENSITIZATION AS RELATED TO DOSAGE OF ANTITOXIN IN MAN AND ANIMALS.*

WILLIAM H. PARK, L. W. FAMULENER, AND E. J. BANZHAF.

(From the Research Laboratory, Department of Health, New York City.)

The results of a series of tests carried out by Lewis[1] showed that 2 units of antitoxin contained in 0.05 c.c. of horse serum given one hour previously protected rabbits which received about 10 fatal doses of diphtheria toxin. Both injections were given intravenously. When rabbits were sensitized by a previous injection of either normal or antitoxic horse serum, several times as much antitoxin was required. The amounts were not graded sufficiently to state the results more accurately.

Lewis recognizes that the experiments are limited in number and suggests that the curve of absorption of antitoxin in man as developed by J. Henderson Smith[2] be elaborated to cover the case of human beings who have received previous injections of normal or antitoxic horse serum. During the course of our studies upon the influence of protein concentration upon the absorption of antitoxin (see p. 338) we had an opportunity to make observations upon men which have a direct bearing upon this question. Also, further tests of like nature were carried out on goats. The results obtained from our tests have been plotted on charts for ease of comparison.

EXPERIMENTS WITH MEN.

The data pertaining to the men are briefly submitted, as follows: S. received on February 11, 1913, an initial injection subcutaneously of 10,000 units diphtheria antitoxin in 4.5 c.c. volume of a preparation containing 17.2 per cent of pseudoglobulin. Two months later he received a second injection of a preparation containing the same number of antitoxic units and amount of pseudoglobulin but diluted to 9 c.c. volume. This was followed by a moderate local reaction. F. received subcutaneously on February 6,

* Received for publication January 15, 1914.

[1] *Jour. Exper. Med.*, 1912, 16, p. 216.

[2] *Jour. Hyg.*, 1907, 7, p. 205.

1913, an initial injection of 10,000 units of diphtheria antitoxin in 9 c.c. volume of a preparation containing 8.6 per cent of pseudoglobulin. His second injection was given 2 months later. It contained the same number of units of antitoxin and the same amount of pseudoglobulin, but in a volume of only 4.5 c.c. The local reaction was moderate in amount.

The above details are given for purposes of accuracy. The protein concentration and the volume of fluid are believed by us to have practically no appreciable effect. The results of these tests are represented graphically in Chart 1 upon a 100 lb. basis.

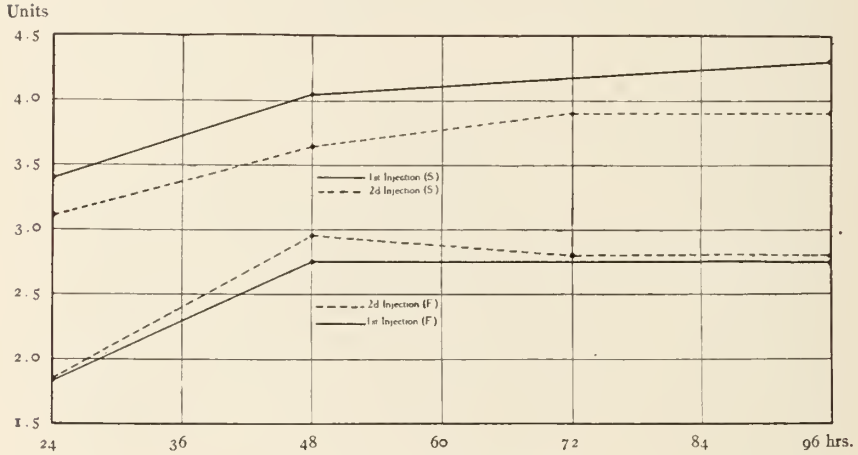

CHART 1.—Comparative tests in man.

EXPERIMENTS WITH GOATS.

Goat 5 received the first injection subcutaneously on October 17, 1912, consisting of 10,000 units of diphtheria antitoxin in a volume of 10 c.c. fluid containing 29.2 per cent of pseudoglobulin. Its weight was 47.5 lbs. Slightly over 7 months later, the same animal received an injection of an antitoxic preparation corresponding in every particular with the first injection. At this time its weight had increased to 57 lbs. Chart 2 shows the results of both tests in curves with unit value per cubic centimeter of serum (goat) based upon 50 lbs. weight.

Goat 25, weight 47.5 lbs., was given subcutaneously a sensitizing dose of 10 c.c. normal horse serum on March 19, 1913. On

May 21, 1913, the animal was given a second injection subcutaneously of a diphtheria antitoxin preparation containing 10,000 units in 2.7 c.c. volume with 29.2 per cent of pseudoglobulin. Two animals weighing 54 lbs. and 46.5 lbs., respectively, which

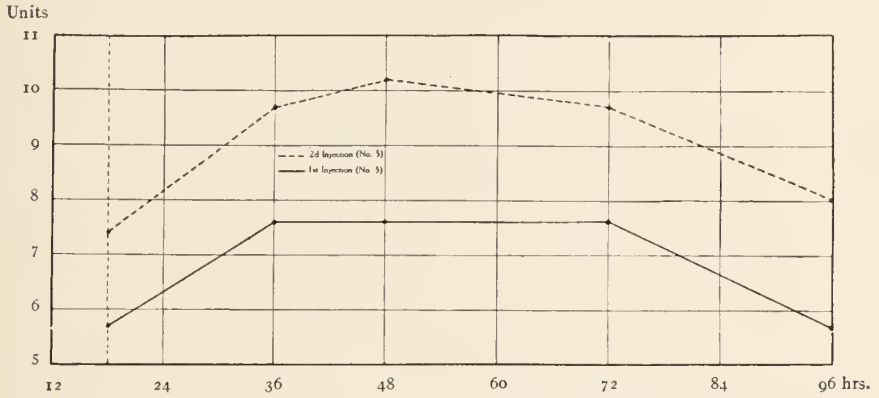

CHART 2.—Absorption curves of first and second injections of antitoxin in Goat 5.

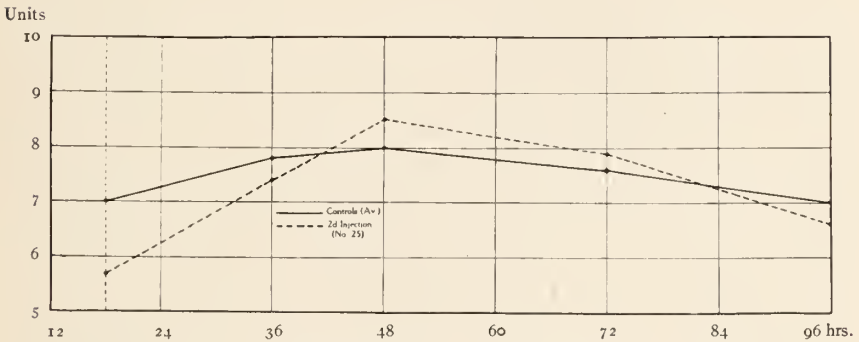

CHART 3.—Average antitoxin content in 2 goats receiving first injections, and of Goat 25 receiving a second injection.

had received primary injections of an antitoxic preparation corresponding in unit strength, volume, and protein concentration with that given to Goat 25, were used as controls. The unit values of the serum samples from each animal were brought to the 50 lb. basis, and in case of the 2 controls the average taken, and then comparative curves were plotted as shown in Chart 3.

SUMMARY AND CONCLUSIONS.

The results obtained in 2 men and in 2 goats showed no appreciable difference in the absorption curves of antitoxin before and after sensitization. The variations that occurred in the cases appeared to be due to the inherent individual characteristics of the persons and animals injected and not to the sensitization.

We conclude that the large amounts of antitoxin injected in the treatment of diphtheria are neither bound nor destroyed appreciably by any globulin antibodies present in the blood of those previously injected. The same quantity of antitoxin is therefore indicated in the treatment of diphtheria whether the case has or has not received a previous injection of horse serum or globulins.

PROTECTIVE ENZYMES, CYTOTOXIC IMMUNE SERA, AND ANAPHYLAXIS.*

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The announcement by Abderhalden of his theory of protective enzymes and his practical application of this theory to the diagnosis of pregnancy has stimulated a large amount of investigation, the general aim of which is to establish the theory as a basis for the explanation of the pathology of various diseases. The large amount of literature[1] which has already accumulated indicates not only how eagerly the method has been investigated as a practical aid in the diagnosis of pregnancy, but also the way it has been utilized in the study of other conditions, as malignant tumors,[2] disturbances of internal secretion[3] and in nervous and mental diseases.[4]

The value of these applications, representing, as they do, efforts at immediate practical use, can be determined only by the statistics of cumulative work and may be left for the present to the efforts of the clinician using the exact methods of the laboratory. The extension of the theory, however, to include such diverse conditions as pregnancy, malignant growth, and mental disease, suggests that for such general application, the underlying principle should be definitely established.

That the theory of protective enzymes applies to fats and carbohydrates as well as to proteins is the view of Abderhalden, who, after injecting these various substances into animals, found in the serum respective ferments for each. Such a demonstration is of unusual interest to both physiologist and pathologist, and if definitely confirmed, it is a most important addition to our knowledge of the methods by which the body protects itself against the parenteral introduction of foreign material. But if the theory is to be applied to explain the pathology of disease, it is essential to demonstrate specificity of reaction to proteins of diverse composition or at least to the peculiar proteins of certain organs. Abderhalden's views[5] as to specificity have been offered chiefly in his papers on the test for pregnancy. In connection with this test, he discusses various odd results: (1) 5 cases of eclampsia, the serum of 4 of which digested liver tissue and that of the fifth, thyroid tissue; (2) the serum of a case of nephritis of pregnancy which digested liver and kidney tissue; (3) that of a case of myxedema, with suspected pregnancy, which digested thyroid tissue but not placenta and liver; and (4) a case of Basedow's disease, the serum of which digested thyroid and ovarian

* Received for publication November 26, 1913.

[1] For the general literature see Abderhalden, *Schutzfermente des tierischen Organismus*, Berlin, 1912; *Abwehrfermente des tierischen Organismus*, 1913.

[2] Abderhalden, *München. med. Wchnschr.*, 1913, 60, p. 411; also Markus, *Berl. klin. Wchnschr.*, 1913, 50, p. 776.

[3] Munzer, *ibid.*, p. 777.

[4] Fauser, *Deutsch. med. Wchnschr.*, 1912, 38, p. 2446; *ibid.*, 1913, 39, p. 304; also *München. med. Wchnschr.*, 1913, 60, p. 584.

[5] *München. med. Wchnschr.*, 1913, 60, p. 462.

tissue, but not liver and testicle. These side reactions may, however, it is suggested, represent the action of ferments which have developed as a response to disease of the liver, ovary, and thyroid, respectively, or it may be that the sera of certain diseases (cancer, febrile disease, and diseases accompanied by exudate) contain, in large amounts, the products of tissue destruction. It is definitely stated, however, that after the parenteral injection of some blood-foreign materials, as, for example, carbohydrates, the ferments resulting are not always strongly specific. They may digest not only the material injected, but also closely related combinations of the same class. This lack of specificity would appear, however, to apply more especially to the carbohydrates and not to proteins, for, by the optic method, the effect of a specially prepared protein serum upon closely related proteins failed to show a general proteolytic action.

On the basis of Abderhalden's general theory that the presence of the chorionic cells, or the products of their destruction, in the blood stream is responsible for the development of the protective ferment of pregnancy, one would expect ferments to appear in the blood-stream following the injection of kidney, liver, foreign serum, or other protein, which would be specific for the cell, serum, or protein injected. Some experimentation on this point exists. Thus Abderhalden[1] found that the subcutaneous or intravenous injection of placenta or placenta peptone into animals (including males) caused the development in the blood serum of a ferment capable of digesting human placenta. Positive results were obtained in each of 3 dogs injected twice with human placenta peptone and tested after 8 days; also in 6 rabbits injected 4 times with the fresh juice of human placenta and tested after 6 days; and likewise in 2 guinea-pigs receiving extract of guinea-pig placenta. In making the injection the intravenous method was used and in many instances the injections were made on successive days. Such experiments, in some of which the serum of animals receiving guinea-pig placenta was tested with human placenta, naturally bring up the question of specificity and also the question whether or not the protective enzyme has any relation to the active body in a cytotoxic immune serum.

It was to test these two points that the present investigation was undertaken, and as one of us had had considerable experience with the production of nephrotoxic immune sera, and was moreover desirous of applying the theory of protective enzymes to the study of the problems of nephritis, the work was limited to a study of the reactions following the injection of kidney substance.

Aside from the observations of Abderhalden, little evidence based on experimental study is at hand. Heilner and Petri[2] found that experimental hematoma in the rabbit causes the appearance in the serum, after 6 hrs., of bodies capable of digesting rabbit's liver, placenta, muscle, and coagulated blood serum. Petri[3] also injected rabbits subcutaneously with their own serum and the serum of other rabbits and found, after 15 mins. to 48 hrs., that the serum of rabbits so treated contained fer-

[1] *Op. cit.;* see also *Deutsch. med. Wchnschr.*, 1912, 38, p. 2160, and *Ztschr. f. physiol. Chem.*, 1912, 77, p. 249.

[2] *München. med. Wchnschr.*, 1913, 60, p. 1530.

[3] *Ibid.*, 1913, 60, p. 1137.

ments for coagulated serum, muscle, and liver of the rabbit. In one experiment 0.1 c.c. of rabbit serum caused the appearance of ferments in 6 hrs. Similar observations were made on man with the injection of human serum, with like results. Frank, Rosenthal, and Biberstein[1] injected rabbits and dogs intraperitoneally and subcutaneously with 5 gm. of sheep's kidney. After 36 hrs. the serum digested the liver and kidney of the rabbit and the sheep. In a second similar experiment serum drawn after 2 days digested human placenta, sheep's kidney, and chicken liver; in a third experiment, human placenta was digested, but not sheep's kidney; in a fourth, both were digested. When the sheep's kidney was injected a second time after an interval of several days a serum was obtained which digested only sheep's kidney. This they consider a selective ferment for sheep's kidney. In another communication, Frank and Rosenthal[2] compare the hemolysis and precipitin tests of a sheep-rabbit system with the dialysis method of Abderhalden and reach the conclusion that the protective enzymes have no relation to the bodies (amboceptor, etc.) concerned in immunity reactions. Steising,[3] after studying, in connection with Abderhalden's dialysis method, inactivated sera with and without addition of guinea-pig and human complement, comes to the opposite conclusion and suggests that the so-called protective ferments belong in the group known by immunologists as lysins. Abderhalden[4] states that amboceptor and complement play no part in the ferment theory and that Steising's positive results were due to the large amount of ninhydrin reacting substances which are frequently found, on account of peculiarities of digestion, in the serum of herbivorous animals.

Abderhalden[5] also presents evidence to show that intravenous injection into a rabbit of 3 c.c. of hemolyzed rabbit's blood causes the serum of the injected animal to digest rabbit's red cells, but not rabbit's serum proteins.

I. NEPHROTOXIN AND PROTECTIVE FERMENT.

Methods.—Abderhalden's method of demonstrating the protective ferment of pregnancy[6] is now so generally known that it is not necessary to give his methods in detail. Of the two, the optic and the dialysis methods, the latter has been used in this investigation. Our procedure has been as follows:

In the work with kidney protein immunity, the usual method of developing a cytotoxin was used. Dog's kidneys were washed free of blood by passing, under ether anesthesia, many liters of normal salt solution through the abdominal aorta. Under aseptic conditions the renal cortex was finely ground, mixed with salt solution, and injected into the peritoneal cavity of rabbits in amounts of 2–3.5 gm. For each treatment the material was freshly prepared. Some animals received a single injection; others 2–5 injections and after varying periods of time the animals were bled and the digestive action of the serum upon kidney and other tissues was tested. For the dialysis, Schleicher and Schüll diffusion sacs (Nos. 579 and 579A) which had been tested against peptone and normal serum were used. The kidney substance for the digestion tests was obtained in the same way as that used for the injections and after boiling and testing according to Abderhalden's technic, it was broken up into small

[1] *Ibid.*, 1913, 60, p. 1594.
[2] *Ibid.*, 1913, 60, p. 1425.
[3] *Ibid.*, 1913, 60, p. 1535.
[4] *Ibid.*, 1913, 60, p. 1641.
[5] *Ibid.*, 1913, 60, p. 1703.
[6] Williams and Pearce, *Surgery, Gynecology and Obstetrics*, 1913, 16, p. 411.

granular masses, again tested, and then placed in distilled water to which chloroform and toluene had been added and was kept in the refrigerator at a temperature a little above freezing. This material was boiled and tested before each series of observations. When the amount of serum obtained was sufficient, at least 8 sacs were used in each experiment, thus, serum of injected rabbit, (1) alone, (2) plus dog's kidney, and (3) plus dog's liver; normal rabbit serum, (4) alone, (5) plus dog's kidney, and (6) plus dog's liver; (7) kidney alone, and (8) liver alone. In some instances, when the amount of serum was small, as in the case of successive bleedings of the same animal, all these tests were not possible. The amount of serum used was 1.5 c.c. and of liver or kidney tissue, 1 gm. The sacs were kept at a temperature of 37.5° C. for 18–20 hours. The ordinary 50 c.c. centrifuge tube was found to be a convenient container for these sacs, as, in addition to the sac, it holds 20 c.c. of distilled water comfortably, with the sac in an upright position, and with the fluid within and without the sac at about the same level. Toluene was used freely within both tube and sac. For the demonstration of the products of digestion, ninhydrin (triketohydrindenhydrate) was used according to Abderhalden's directions.

In the first series of experiments, rabbits received a single injection of renal tissue and the serum of each was tested at various intervals after injection. The satisfactory results in this group are shown in Table 1.

TABLE 1.

EXPERIMENTS WITH SERA OF RABBITS RECEIVING A SINGLE INJECTION OF RENAL TISSUE.

EXPERIMENT NO.	RABBIT NO.	DAYS AFTER INJECTION	KIDNEY SERUM			NORMAL RABBIT SERUM			DOG'S KIDNEY ALONE	DOG'S LIVER ALONE
			Alone	With Dog's Kidney	With Dog's Liver	Alone	With Dog's Kidney	With Dog's Liver		
1.....	2	2	†	++	.....	.....	.....	.....	o	.....
2.....	2	5	†	++	.....	†	†	.....	o	.....
3.....	8	5	+	++	++	.....	.....	.....	o	o
4.....	3	6	o	++	.....	.....	.....	.....	o	.....
5.....	9	6	†	+	+	o	o	o	o	o
6.....	7	8	o	o	o	o	o	o	o	o
7.....	1	10	†	+	.....	o	o	.....	o	.....
8.....	10	18	+*	o	o	o	o	o	o	o
9.....	1	33	†	+	+	o	o	o	o	o
10.....	3	37	†	++	+	†	†	†	o	o

† indicates faint reaction; +, well marked blue color; ++, deep blue color; o, no change.
* Positive result in this instance was due to a faulty sac.

The results differ widely. In Experiments 6 and 8 no digestive enzyme could be demonstrated 8 and 18 days, respectively, after injection. In Experiments 1, 2, 4, and 7, in which the amount of serum was insufficient for control tests with liver, an action on kidney tissue was evident, but in two of these (Experiments 1 and 7) the serum alone reacted faintly to ninhydrin. In Experiments 3, 5, 9, and 10, positive results were obtained with liver as well as kidney, and with one exception they were of the same degree of

intensity. In Experiments 2 and 10 the control normal sera gave faintly positive reactions; in Experiments 5, 6, 7, 8, and 9, they were negative. When an animal was used more than once the same reaction was obtained at each bleeding (see Experiments 1 and 2, 7 and 9, 4 and 10). The periods after injection at which positive results were obtained varied from 2 to 37 days.

In analyzing these results, one must keep in mind the many difficulties of the technic. The sacs go wrong so frequently that they must be tested against peptone and serum again and again and especially whenever an unexpected result occurs. Difficulty also arises from the presence of even small traces of hemoglobin in the serum and this complication cannot always be avoided, no matter how carefully the serum is collected. Again, as Abderhalden[1] has pointed out, and also McCord,[2] ninhydrin reacting substances, on account of the peculiarities of digestion in herbivorous animals, are frequently present in the normal serum of the rabbit. These possibilities of error, peculiar to the method, demand great caution in the reading of results, and that they are real difficulties is shown by the fact that of 23 tests made, only 10 were sufficiently free of possible error to be considered as satisfactory for insertion in Table 1. The greatest difficulty in interpretation has occurred naturally in those experiments in which the serum itself gave a reaction. Our rule has been, if such reaction is faint as compared to a strong reaction in tube containing serum and kidney, to consider the results as satisfactory in a comparative sense. If, however, the 2 tubes show reaction of nearly equal degree, the entire experiment is considered worthless. This procedure is in accord with the principles of the technic as laid down by Abderhalden,[3] and seems justified by our own experience with normal control sera occasionally giving a faint positive reaction (see Experiments 2 and 10). With the exception of such comparable reactions all tests in which discordant results occurred have been ruled out. Even with these precautions, we present the above table with some hesitation, because of our doubt of the value of a method which does not give clean-cut controls.

[1] *Op. cit.*

[2] *Surgery, Gynecology and Obstetrics*, 1913, 16, p. 418.

[3] *Op. cit.*

However, as presented, our results indicate that the injection into the rabbit of blood-free kidney of the dog results in the production of a ferment capable of acting upon dog's kidney *in vitro*. This ferment appears in the serum in our experience within 48 hrs. and is still present after 37 days. Inasmuch, however, as it acts with equal power upon dog's liver also, it cannot be considered as specific for the kidney. Occasionally the injection of kidney does not yield a ferment.

As Frank, Rosenthal, and Biberstein[1] report that 2 injections of kidney substance cause the appearance in the serum of a ferment having a selective action on the kidney, we continued our observation on animals receiving 2–5 injections of renal substance.

Here again we have met with many difficulties. Discordant results have been frequent; thus negative results have been obtained after 2–3 injections and positive results after 4–5 injections. When the first negative results after 2 injections were obtained, it was thought that protective enzyme production might be analogous to anaphylaxis, that is, that multiple injections might alter the enzyme production as they do sensitization in anaphylaxis. Later results showed, however, that this view was untenable. Nevertheless, it is difficult to understand why, with similar technic, a serum should be active after 4–5 injections and not always so after 2–3 injections, especially when positive results are readily obtained as a rule after one injection. We have no explanation to offer, but believe that the results in the negative experiments represent some error inherent in the method or some peculiarity in the reacting power of the animals (compare Experiments 6 and 8 of Table 1).

To illustrate this phase of our study the results obtained with the serum of the 2 rabbits receiving the greatest number of injections are presented in Table 2. The injections were made as in the earlier series, and repeated at intervals of 6–7 days. Rabbit 4 was bled 9 days, and Rabbit 12, 8 days after the last injection.

As regards the question of specific action these experiments indicate a more definite action upon kidney than upon liver, and in so far as a small number of observations are of value, support the view that multiple injections tend to a slight selective action.

[1] *Op. cit.*

Another object of this investigation has been to determine whether a serum showing a digestive action on kidney *in vitro* has also nephrotoxic power *in vivo*, that is, to determine the question of the relation between protective enzyme and immune cytotoxin. Inasmuch as it has been shown by earlier workers[1] that a nephrotoxic serum may be readily produced by three or more injections of renal substance, the present study has necessarily been limited to the serum of animals which after a single injection developed the power of digesting kidney tissue *in vitro*.

TABLE 2.

SERA OF RABBITS RECEIVING MULTIPLE INJECTIONS OF DOG'S KIDNEY.

RABBIT NO.	NO. OF INJECTIONS OF DOG'S KIDNEY	KIDNEY SERUM			NORMAL SERUM			DOG'S KIDNEY ALONE	DOG'S LIVER ALONE
		Alone	With Dog's Kidney	With Dog's Liver	Alone	With Dog's Kidney	With Dog's Liver		
4.....	4	o	+	†	o	o	o	o	o
12.....	5	†	++	+	†	†	†	o	o

Such sera have been injected intravenously into dogs and the urine of these animals examined for albumin and casts and finally their kidneys examined for histological changes. The sera selected were those of Rabbits 1, 8, and 9 (see Table 1), all of which were active against kidney tissue, 5, 6, and 33 days, respectively, after a single injection of kidney. These sera given intravenously to dogs in doses of 1–2 c.c. per kilo of body weight caused no disturbance of the kidney. The urine was always free of albumin and the kidneys normal histologically. It would appear, therefore, that the so-called protective ferment resulting from the injection of kidney is not identical with the active substance of nephrotoxic serum, or at least that if the latter is present, it is in such small amount as not to be demonstrable.

In addition to the above experiments in which dog's kidney was injected into the rabbit, 2 dogs received, respectively, 2 and 3 injections of dog's kidney and their serum was tested against dog's kidney *in vitro*. In the first of these the interval between injections was 5 days and the animal was bled 2 days after the second injection; in the other the intervals were 4 and 6 days, respectively, and the bleeding occurred one week after the third injection. Negative results were obtained. These results are not in accord with those of Abderhalden, who found ferments as the result of injecting dog's kidney into the dog. Comparison, however, is not exactly proper, as Abderhalden injected frequently into the circulation and used the optic method.

In connection with the experiments just described, a few experiments were made with the serum of animals suffering from experimental nephritis. The hypothesis upon which these experiments were based was that in animals with destruction of renal tissue, as that caused by a nephritic poison, protective enzymes might develop

[1] See Pearce, *Univ. of Penna. Med. Bull.*, 1903, 18, p. 557.

in the serum. That this rather far-fetched hypothesis has no basis in fact was shown by negative results with the serum of a rabbit in the fifth day of severe uranium nitrate nephritis and with that of a dog with a chronic nephritis due to multiple injections of uranium nitrate and potassium chromate.

In this connection it may be added that the attempts to apply Abderhalden's test to the clinical study of nephritis have given unsatisfactory results. Deutsch and Köhler[1] in a study of 22 sera from cases of chronic nephritis and amyloid kidney obtained a digestion of kidney tissue in 17; positive results were likewise obtained by the use of the same sera with placenta and thyroid. On the other hand, the serum of menstruating women in six instances also digested kidney. Lampé and Popazolu,[2] who studied the serum of several individuals with nephritis, found no evidence of specific action on kidney tissue. In so far as the problems of nephritis are concerned, Abderhalden's theory of protective enzymes as a means of investigation would appear therefore to be of little value.

II. Anaphylaxis and Protective Ferments.

The theory that anaphylaxis depends upon a specific ferment developing as the result of the parenteral introduction into the body of a foreign protein has, since the work of Vaughan and his associates in this field, gained wide acceptance. Abderhalden's theory of protective enzymes, altho more comprehensive in its scope, naturally includes the lesser field of anaphylaxis and it is not surprising that early in his studies of the protective enzymes Abderhalden[3] approached the subject of anaphylaxis from the point of view of ferment production as did also, a little later, Pfeiffer and Mita,[4] and Gruber.[5] Within the last year Abderhalden[6] has published some results of the study of anaphylaxis which are of considerable interest.[7] These are as follows: (1) sera from 12 guinea-pigs sensitized to egg-white, when mixed with antigen, showed digestive power by both optic and dialysis (biuret) methods; (2) similar sera, dialyzed alone, showed digestive products in only 1 of 6 sera tested; (3) serum of 6 guinea-pigs taken at intervals of 5 mins. to 1½ hrs. after the second injection (egg-white) and dialyzed gave negative results after 5 and 15 mins., while 4 taken after 30, 45, 60, and 90 minutes, respectively, were positive. In each test the serum (10 c.c.) was dialyzed against distilled water for 16 hrs. at 37° C. and the presence of products of digestion determined by the biuret reaction.

[1] *Wien. klin. Wchnschr.*, 1913, 26, p. 1361.

[2] *München. med. Wchnschr.*, 1913, 60, pp. 1423 and 1533.

[3] *Ztschr. f. physiol. Chem.*, 1909, 61, pp. 199 and 426; 62, p. 243; also *Schutzfermente, loc. cit.*

[4] *Ztschr. f. Immunitätsf.*, 1910, 6, p. 18.

[5] *Ibid.*, 1910, 7, p. 762.

[6] *Ztschr. f. physiol. Chem.*, 1912, 82, p. 109.

[7] Compare also Zunz, *Ztschr. f. Immunitätsf.*, 1913, 17, pp. 241, 265, and 279.

These observations of Abderhalden's, in view of our experience with antirenal sera, led to the study of animals sensitized to a foreign serum.

Methods.—Dogs received in the peritoneal cavity a single injection of 5 c.c. of horse serum and after the lapse of 3 or more weeks the power of their serum to digest fresh and coagulated horse serum was tested both before and after the second, or intoxicating injection, which was always intravenous and consisted of 3–5 c.c. of the same horse serum. In the digestion experiments 2 c.c. of serum were used in each tube, dialysis was allowed for 18–20 hrs. at 37° C., and ninhydrin was used in all tests to detect the presence of the products of digestion.

As in the work with kidney sera, some difficulty was experienced on account of occasional slight hemoglobin staining of the sera and also because control sera sometimes gave as definite a reaction as the sera of treated animals. It is for these reasons that data cannot be given (see Table 3) concerning the activity of each serum both before and after the second injection. For the same reasons observations on several animals are omitted entirely. Whether or not the positive results with normal serum (not here presented) are due to the presence of proteolytic ferments in the serum of normal dogs[1] we will not discuss at this time.

TABLE 3.

DIGESTIVE POWER OF SERUM OF SENSITIZED ANIMALS BEFORE AND AFTER SECOND INJECTION.

BEFORE SECOND INJECTION.

DOG NO.	SENSITIZED SERUM			NORMAL SERUM			HORSE SERUM ALONE
	Alone	With Fresh Horse Serum	With Coagulated Horse Serum	Alone	With Fresh Horse Serum	With Coagulated Horse Serum	
15	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0
13	0	0	+	0	0	0	0
AFTER SECOND INJECTION.							
15	0	0	0	0	0	0	0
11	0	+	0	0	0	0	0
14	0	+	0	0	0	0	0

Table 3 shows the general results with the first group of animals studied. All sera "after shock" were obtained within periods varying from 4 to 10 minutes after the fall in blood pressure, as determined by kymographic tracing, had occurred. The results are fairly uniform, but are not in accord with those of Abderhalden in that negative results were always obtained before the second injection (except once with coagulated serum), and positive results in two of the sera after such injection. The dialysis of serum alone (2 c.c.) never gave positive results. The almost uniformly negative results with sera obtained before the second injection suggests the possibility of impermeable sacs, but as each sac was satisfactorily tested before and after each test with Witte's peptone and with blood serum the explanation is not tenable. There

[1] Compare Pincussohn, *Biochem. Ztschr.*, 1913, 51, p. 107.

is, however, one point of difference between Abderhalden's technic and ours. Abderhalden used the biuret test while we used ninhydrin. In our hands the biuret test has been most unsatisfactory. Another difference lies in the fact that Abderhalden used guinea-pigs sensitized to egg-white, while we used dogs sensitized to horse serum. The negative results before the intoxicating dose indicate that, by the method used, the serum was, contrary to Abderhalden's experience, without specific ferment. On the other hand, if "shock" following the second injection is the result of the digestion of the foreign protein by specific enzymes, the products of such digestion should be found in the serum. Such products ninhydrin failed to demonstrate when the sera obtained after "shock" were dialyzed without the addition of horse serum. The amounts used, however, were small (2 c.c.) and the serum was obtained always within 5–10 mins., while Abderhalden's positive results were obtained with 10 c.c. of serum from blood drawn at intervals of 30 to 90 mins. after the second injection. In a second series of observations, in order to cover these variations, we used larger amounts of serum (10–20 c.c.) obtained from blood drawn at intervals of 30, 60, and 90 mins. after "shock." These larger amounts were dialyzed alone and the dialysate tested with ninhydrin. Here, again, our work has been most unsatisfactory. The serum from the non-coagulable blood, obtained half an hour or more after shock, has in each of 5 experiments been more or less stained with hemoglobin and with every precaution in collecting and centrifuging we have failed to avoid this difficulty.[1] Dialysis of this serum has always given positive results, but in view of the presence of hemoglobin it has been impossible to decide upon their significance. In an attempt to determine whether the positive results in question are due to the presence of hemoglobin or the products of protein disintegration, we have tried to demonstrate the latter directly. We have removed, immediately after centrifuging the freshly obtained blood, all coagulable protein by heat and acetic acid, or by absolute alcohol and zinc chlorid, or, as we have found to be better, by combining both methods. The resulting filtrate evaporated to dryness, brought back by addition of distilled water to the original volume of serum, and neutralized, has usually given a negative reaction in the case of normal dog serum even tho it be slightly hemoglobin-stained. On the other hand, each of 3 sera obtained after shock and tested in this way gave a positive reaction. On the assumption that by this method all coagulable protein had been removed, these positive reactions could have been due only to the presence of the products of protein disintegration.

The method is not, however, without possibilities of error, one being the uncertainty of removal of all coagulable protein and the other being the destruction of ninhydrin in an excess of acetic acid. (This last source of error we have studied carefully and we have found it to be a very definite cause of trouble. It can be avoided, however, by neutralization of the fluid with weak sodium hydrate solution before adding ninhydrin.) These possible errors we believe have been adequately controlled. Nevertheless, our

[1] Serum from blood drawn 5–10 mins. after shock is occasionally hemoglobin-stained, but as a rule is not; after half an hour, however, it has been our experience that there is always a faint tingeing. For this we have no explanation.

results of the study of the serum of the dog after "shock," tho offered as corroborative of Abderhalden's observations on the serum of the guinea-pig after shock, are presented with some hesitation on account of the fact that our method of procedure differs from that of dialysis so widely as to be open to criticism.

In this connection it is of interest to recall that Auer and VanSlyke[1] failed, by direct determination of amino-nitrogen, to find an increased amount of protein cleavage products in the anaphylactic lung of the guinea-pig.

On the whole our results with the serum of anaphylactic animals are very unsatisfactory. The action on fresh horse serum of serum from the dog obtained before "shock" was not demonstrable in 4 experiments, altho in one a positive result was obtained with coagulated horse serum. After "shock," positive results were obtained with 2 of 3 sera in the case of fresh horse serum, but in none with coagulated horse serum. In none of these experiments did the serum (2 c.c.), when antigen was not used, give a positive reaction on dialysis. When, as did Abderhalden, we used larger amounts (10–20 c.c.) of serum obtained $\frac{1}{2}$–$1\frac{1}{2}$ hrs. after shock, positive results were obtained, but as the serum was hemoglobin-stained, the interpretation was doubtful. As, however, positive results were obtained with the filtrates of these sera after removing the coagulable protein, it would seem probable, despite certain possibilities of error, that the serum after shock does contain the products of protein disintegration. Yet the results are not sufficiently definite to afford proof of the view first elaborated by Vaughan and now adopted by Abderhalden that enzymes developing as the result of the parenteral introduction into the body of foreign protein constitute the essential basis of anaphylaxis. Inasmuch, however, as the suggestive results here given are in part comparable to those of Abderhalden, it is quite possible that some refinement of the method or a nearly allied method of greater accuracy may offer definite proof of Vaughan's very important and presumably correct hypothesis. But we are not in general accord with the results obtained by Abderhalden.

[1] *Jour. Exper. Med.*, 1913, 18, p. 210.

Setting aside the question of any relation which our results may or may not have to the theories of anaphylaxis, it is evident from the results in the first three experiments of Table 3 that the injection of a foreign protein is not always followed by the development of enzymes demonstrable by Abderhalden's dialysis method.

SUMMARY.

1. On the basis of Abderhalden's theory of protective enzymes and by the use of his dialysis method it has been shown that the serum of a rabbit receiving a single injection of kidney substance develops the power to digest dog's kidney *in vitro*, but has no effect upon the kidney of the dog when administered intravenously. Thus it would appear that the so-called protective enzymes are not to be classed with the immune cytolysins.

2. The digestive power of the serum which develops after the injection of kidney is not limited to the kidney but acts also upon the liver. This is true after one injection or after 4 or 5 injections. There is some evidence, however, after multiple injections of a tendency to a more definite effect on the kidney than on the liver.

3. A few attempts to demonstrate protective enzymes in the serum of dogs receiving dog's kidney and of animals with experimental nephritis have failed.

4. Attempts to demonstrate protective enzymes in the serum of dogs sensitized to horse serum have not been as successful as those of Abderhalden with the serum of the guinea-pig sensitized to egg-white. Negative results have been the rule before shock, and positive results, difficult of explanation, after shock.

5. Dialysis, alone, of small amounts (2 c.c.) of serum, obtained either before or 5–10 mins. after "shock" in dogs sensitized to horse serum, gives no evidence of the presence of the products of protein disintegration. Larger amounts (10–20 c.c.) taken $\frac{1}{2}$–$1\frac{1}{2}$ hrs. after shock give positive results after dialysis, but the interpretation of these is doubtful on account of the difficulty, under these circumstances, of obtaining serum free of traces of hemoglobin.

CONCLUSIONS.

The results of the injection of renal tissue support Abderhalden's general contention concerning protective enzymes but indicate a lack of specificity. On the other hand, our work with anaphylaxis, while suggestive, is not sufficiently definite to be used in support of the theory that the essential mechanism of anaphylaxis can be explained on the theory of the development of a protective enzyme. Finally, we wish to state frankly, that on account of the many difficulties which the technic of this method presents—and especially because of the frequent presence of ninhydrin reacting substances in the serum of normal animals—thus rendering exact control observation difficult, these results are presented with some hesitation. Moreover, without desiring to detract in any way from the importance of the underlying principle of Abderhalden's theory of protective enzymes as exemplified by his work on pregnancy, we urge caution as to hasty attempts to apply this theory as a general explanation of widely diverse conditions of altered physiology.

THE IMMUNOLOGICAL RELATIONSHIP OF HORDEIN OF BARLEY AND GLIADIN OF WHEAT AS SHOWN BY THE COMPLEMENT FIXATION, PASSIVE ANAPHYLAXIS, AND PRECIPITIN REACTIONS.*†

THE BIOLOGICAL REACTIONS OF THE VEGETABLE PROTEINS. IV.‡

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There are numerous observations in the literature on biological reactions with extracts of vegetable substances, showing that such extracts, containing mixtures of the soluble vegetable proteins of the plant material employed, are capable of acting as antigens which will react with, and incite the formation of, antibodies demonstrable by precipitin, complement fixation, and anaphylaxis reactions. Much of this literature is reviewed in the first article of this series and it concerns especially the precipitin reaction. None of this literature refers to experiments performed with isolated proteins, with the exception of casual observations by Jacoby and by Osborne, Mendel, and Harris, that the serum of animals immunized with purified ricin gives precipitates with ricin. At that time we found no publications on the occurrence of complement fixation reactions with vegetable antigens, except observations by Dunbar on pollens. Since then this subject has received some consideration, the literature of which is here reviewed.

Ballner[1] reports that strong and specific complement fixation reactions can be obtained with solutions made by extracting the ground grains, etc., with physiological salt solution, heating the extract 5 hrs. at 56° C., and then filtering. He describes these extracts as giving the chemical reactions characteristic of albumoses. The serum of rabbits immunized with these solutions gave fixation reactions indicating a distinct

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‡ The preceding articles of this series are: I. Wells and Osborne, *Jour. Infect. Dis.*, 1911, 8, p. 66; II. Wells and Osborne, *ibid.*, 1913, 12, p. 341; III. White and Avery, *ibid.*, 1913, 13, p. 103.

[1] *Sitzungsber. kaiserl. Akad., Wien*, 1910, 119, Abt. 3, p. 17.

"group specificity." Thus, anti-wheat serum gave reactions with wheat extract in dilutions of 1–20,000, and with rye extract 1–10,000; reactions were also given with extracts of barley and oats in considerably greater concentration, but extracts of rice and corn reacted with wheat antiserum only in extreme concentrations, as did also the extracts of peas and lentils. Pea antiserum was very specific, reacting with pea extract at 1 to 40,000 dilution, and with lentil extract only in dilution of 1 to 50. Since these extracts used by Ballner contained several of the proteins present in the materials extracted, it is impossible to determine which of these caused the reactions.

Dunbar,[1] in his work on "hay fever," found that various pollens can be differentiated from one another and from other parts of the same plant by the complement fixation reaction.

Wendelstadt and Fellner[2] found that extracts of leaves of various fruit trees, as well as extracts of leaves of cereals, gave unsatisfactory results by the complement fixation reaction, but that simple saline extracts of the seeds of beans and peas gave positive reactions of certain well defined degrees of specificity. Antiserum for extracts of the beans of *Vicia faba* reacted with similar extracts from *Vicia sativa* and *Pisum sativum*, but not with those of *Phaseolus vulgaris* or *Phaseolus multiflorus;* antiserum for seeds of *Pisum sativum* reacted with extracts of *Vicia sativa* and *Vicia faba*, but not with those of *Phaseolus;* antiserum for *Phaseolus grandiflorus* reacted with extracts of *Phaseolus vulgaris*, but not with extracts of *Pisum sativum*, *Vicia sativa*, or *Vicia faba*. However, the reactions with the heterologous extracts were always less strong than with the homologous extracts, except in the case of *Phaseolus*. These results may be compared with those which they obtained by the precipitin reaction. A very strong precipitating antiserum for extracts of the seeds of *Vicia faba* gave almost as strong precipitating reactions with extracts of *Pisum sativum* and *Vicia sativa* as with extracts of *Vicia faba*, but none with extracts of *Phaseolus vulgaris* or *grandiflorus*, thus agreeing with the complement fixation reaction. Furthermore, experiments by means of passive anaphylaxis showed that rabbit antiserum for extracts of *Pisum sativum* made guinea-pigs sensitive to *Vicia faba*, but not to either variety of *Phaseolus*. Peculiar results were obtained with the serum of rabbits immunized with extracts of green leaves; thus, antiserum for pear leaves reacted more strongly with extracts of plum and peach leaves than with extracts of pear leaves themselves; antiserum for barley leaves reacted strongly with the extracts of leaves from wheat and rye, but only slightly with extracts of barley leaves. Feeding rabbits for some time with peas, corn, or potatoes did not cause the appearance in their blood of appreciable amounts of antibodies causing either precipitin or complement fixation reactions.

According to Sauli[3] the conglutination reaction gives sharper results with antisera for vegetable proteins than does the precipitin reaction, and he therefore applied this reaction to a number of plant extracts. He found that antiserum for extracts of seeds of *Brassica rapa rapifera*, which gave practically no reaction with extracts of seeds of *Pisum sativum* or *Trifolium pratense*, gave about as strong a reaction with extracts of the seeds of *Brassica napus rapifera* as with the homologous extract; in general, this serum gave strong reactions with extracts of seeds from all plants of the family *Cruciferae*, but very weak or none at all with *Papilionaceae*, thus showing a strong group reaction for botanically related species. Antiserum for *Vicia faba*

[1] *Ztschr. f. Immunitätsf.*, 1910, 4, p. 740.

[2] *Ibid.*, 1910, 8, p. 43.

[3] *Ibid.*, 1911, 9, p. 359.

aquina gave an almost equally strong reaction for *Vicia faba atropurpurea* but considerably weaker for *Pisum sativum*.

An interesting observation is described by Pick,[1] to the effect that an immune serum containing precipitins for edestin has no effect upon edestin crystals suspended in it, but if sufficient salt is added to cause a little of the edestin to go into solution, immediate precipitation occurs.

Recently White and Avery,[2] working with the same preparations that have been used in other experiments described in this series of articles, obtained a precipitin for edestin which gave strong reactions with solutions of edestin diluted to 1 to 10,000, but did not react with gliadin in even twice as great a concentration; specific complement fixation was also obtained with the serum of rabbits immunized with edestin, but no reaction was obtained with the same serum and gliadin.

Chapman,[3] in an address, states briefly that experiments have been performed in his laboratory with extracts made with 10 per cent NaCl solution from seeds of 15 species of *Acacia*, the extracts being heated at 55° C. for 3–6 hours for sterilization. Extracts of 11 out of the 15 different species of seeds gave precipitates with normal serum. Extracts of *Acacia pycnantha* were used for immunizing, and the resulting antiserum gave precipitin reactions with extracts of all the acacias, but none with extracts of leguminosae (*Pisum*, *Vicia*, *Phaseolus*) nor of wheat, oats, or other unrelated forms.

Lusini[4] reports the production of specific precipitins for *Lytta vesicatoria*, *Smilax officinalis*, *Althaea officinalis*, *Jatcorrhyza palmata*, and *Digitalis purpurea;* uncertain results were obtained with *Aloes*, and negative results with *Rheum palmatum* and *Picraene excelsa*. This work was done with the object of applying the immunity reactions to the identification of botanical drugs.

To complete the review of the literature with indirectly related observations, we mention that Rosenblatt-Lichtenstein[5] found that immunization with *Algae* of different species produces quantitatively specific agglutinins. Gallio-Valerio and Bornand[6] immunized rabbits with extracts of *Agaricus muscarina Linn.* and obtained precipitins which differentiate this from *Boletus edulis* and other mushrooms. Thornton[7] also states that plant and animal cells have opposite electrical charges, animal cells being negative and plant cells positive, but that the contact of the two types of cells does not result in a mutual discharge, from which fact he concludes that the charge is not in or on the cell membranes, but in the cytoplasm.

There is much evidence in support of the hypothesis that the precipitin, agglutinin, complement fixation, and anaphylaxis reactions all represent the interaction of one and the same specific immune body with its corresponding antigen, the different reactions being merely different methods of demonstrating the presence of

[1] Kolle and Wassermann, *Handbuch d. path. Mikroorg.*, 1912, 1, p. 689; the work is credited to Obermayer and Pick but no reference is cited.

[2] *Jour. Infect. Dis.*, 1913, 13, p. 103.

[3] *Proc. Linnean Soc.*, New South Wales, 1910, 35, p. 549.

[4] *Atti. R. Fisiocrit.* (Siena), 1912, 219, p. 147.

[5] *Arch. Anat. u. Physiol.*, 1912, Phy. Abt., p. 415.

[6] *Ztschr. f. Immunitätsf.*, 1913, 17, p. 180.

[7] *Proc. Roy. Soc.* (B), 1910, 82, p. 638.

this antibody. Observations have been made, however, which are not in harmony with this simple interpretation, so that at present this view cannot be considered as established.

EXPERIMENTS.

Since hordein from barley (*Hordeum vulgare*) and gliadin from wheat (*Triticum vulgare*) or from rye (*Secale cereale*) are similar in their physical properties, in the proportion of their products of hydrolysis, and have been shown to be closely related to one another by the anaphylaxis reaction,[1] we have undertaken to study the complement fixation, the precipitin, and the passive anaphylaxis reactions exhibited by these physically and chemically unique proteins.

In all our experiments the following methods were used:

A. *Passive anaphylaxis.*—The serum or defibrinated blood from an immunized rabbit, in doses varying from 0.5 to 3.0 c.c., was injected into the peritoneum of guinea-pigs (200–300 gm.) and the antigen was injected into these in doses of 0.1 gm. (or 1 c.c. fluid antigen) after an interval of from 24 hrs. to 4 days.

B. *Precipitin test.*—Different quantities of the protein to be tested were used with 0.1 c.c. of the unheated serum of the immunized animal. Similar series of experiments with the serum of normal animals of the same species, and corresponding amounts of antigen, were used as controls.

C. *Complement fixation.*—The antigen was used in different amounts: of the antiserum, 0.1 c.c.; of the complement (guinea-pig), 0.05 c.c.; of sheep corpuscle amboceptor, twice the unit amount; of 5 per cent washed sheep corpuscles, 1 c.c. For controls, similar series were run with normal serum; and also the usual controls of antigen and amboceptor were made. Usually the serum was inactivated, but this seemed to make no difference; in final tests inactivated serum was always used.

Some difficulty was encountered in the production of the antiserum on account of the relatively slight solubility of the proteins, and especially of the gliadin. In the earlier work the intravenous method of injecting was used, but on account of the loss of animals through embolism, anaphylactic shock, etc., this method was finally dropped and the intraperitoneal route used entirely, as this seemed to give just as potent an antiserum, without loss of animals.

ABSTRACT OF PROTOCOLS.

Experiment 1.—Gliadin, wheat. During a period of 23 days a rabbit was given six intravenous injections containing in all 1.2 gm. of gliadin dissolved in 0.1 per cent

[1] See preceding paper, II, *Jour. Infect. Dis.*, 1913, 12, p. 341.

NaOH, but it died suddenly immediately after the last injection. (In all our experiments the vegetable protein solutions were prepared just before injecting.) Blood drawn on the sixteenth and twenty-third days gave no precipitin reaction with wheat gliadin in dilutions varying from 1–400 to 1–4,000.

Experiment 2.—Gliadin, wheat. A rabbit received during the course of 7 weeks 10 intravenous injections, containing in all 2 gm. of gliadin dissolved in 0.1 per cent NaOH, the dates of the injections being as follows: February 13, 19, 23, and 26, March 1, 8, 9, and 22, and April 1 and 10. The rabbit died on April 30 in poor condition.

Precipitin tests were made with dilutions of 1–400 up to 1–400,000, but negative results were obtained with blood drawn on the following dates: March 1, 8, and 16.

Complement fixation tests were made on April 13, and positive reactions obtained with dilutions of 1–400 to 1–4,000, the only dilutions tried.

Passive anaphylaxis experiments were tried on April 16 and 23; each time 3 guinea-pigs were given an intraperitoneal injection of the rabbit's blood (from 1 to 3.5 c.c. to each animal), but none of these animals reacted to gliadin injected 48–72 hrs. later.

Experiment 3.—Gliadin, wheat. During 27 days a rabbit received 8 intraperitoneal injections containing a total of 4.7 gm. of wheat gliadin dissolved in 0.1 per cent NaOH, spaced as follows: June 19, 24, 27, July 1, 5, 8, and 15. On July 22 blood was drawn and the complement fixing power determined. Positive reactions were obtained with wheat gliadin in dilutions up to 1–10,000. Negative results were obtained with hordein and rye gliadin in dilutions of 1–2,000. As another rabbit at this time was furnishing a serum of much higher power, nothing further was done with this animal.

Experiment 4.—Gliadin, wheat. A rabbit received injections containing gliadin on the same dates and with the same amounts as in Experiment 3. Ten days after the last injection it was bled to death, and the serum used for experiments. On July 22 a sample of blood was drawn and found to give a positive complement fixation reaction with wheat gliadin in a dilution of 1–100,000, while negative reactions were given with hordein, rye gliadin, wheat "proteose," and malt "proteose" in dilutions of 1–2,000. Positive complement fixation was given with wheat gliadin on July 29, with dilutions of 1–20,000, while no reactions were obtained with the above-mentioned heterologous proteins at 1–2,000. (More concentrated solutions cannot be used safely as a routine practice, because stronger solutions of these vegetable proteins often yield precipitates with serum, but occasionally, as will be noted, we have been able to make tests with strengths as great as 1–400.) Precipitin tests were made with this serum, but no precipitin reaction was obtained with wheat gliadin in a dilution as low as 1–1,000.

A test of passive anaphylaxis was made by injecting 3 c.c. of this serum (intraperitoneally) into each of 5 guinea-pigs. Forty-eight hours later 0.1 gm. wheat gliadin was injected into each with negative results.

Experiment 5.—A 5 per cent solution of hordein, dissolved in 0.1 per cent NaOH solution, was injected into the ear vein of a rabbit; 7 injections, containing a total of 2.63 gm. of hordein, were thus given during 26 days. The animal died suddenly after the last injection. Serum obtained on the twenty-third day, after 5 injections had been made, gave no precipitin reaction with hordein in dilutions of 1–2,000 or greater.

Experiment 6.—Hordein was injected as in Experiment 5, and the rabbit was bled to death 17 days after the last injection. Precipitin tests were made on the twenty-third, thirty-fourth, and forty-fifth days, all being negative with hordein itself, in dilutions varying from 1–1,000 to 1–800,000, as well as with wheat gliadin, rye gliadin, wheat "proteose," and malt "proteose."

On the forty-second day the serum gave a positive complement fixation with hordein in dilutions up to 1–400,000, but no reaction with the above-mentioned heterologous proteins in dilutions of 1–2,000 and up. Again, on the forty-fifth day, positive results were obtained in dilutions of 1–600,000 of hordein, but with the heterologous proteins negative results were given in dilutions of 1–2,000 and up. Negative results were also obtained when an attempt was made to produce passive anaphylaxis as follows: On the thirty-fifth day blood was drawn and injected intraperitoneally into 6 guinea-pigs, the doses ranging from 0.5 to 2.5 c.c. Two to three days later 0.1 gm. hordein was injected intraperitoneally with negative results. Negative results were also given by 8 other guinea-pigs receiving 2 c.c. of serum drawn on the forty-fifth day, and, from 2 to 5 days later, an intraperitoneal injection of 0.1 gm. hordein.

Experiment 7.—Hordein was given to the amount of 3.5 gm. in 7 doses during a period of 22 days. Seven days after the last injection a sample of serum gave positive complement fixation in dilutions up to 1–200,000 with hordein, but not with wheat and rye gliadin in dilutions of 1–2,000. No other experiments were performed with this animal.

Experiment 8.—Injections were made as in Experiment 7. A sample of serum obtained on the twenty-ninth day gave a positive complement fixation reaction in dilutions up to 1–300,000 with hordein, but none with wheat or rye gliadin, wheat "proteose," or malt "proteose." On the thirty-second day the serum gave a positive fixation reaction with hordein in dilutions up to 1–500,000, but not beyond. No reactions were given with the heterologous proteins in dilution of 1–2,000. No precipitin reaction was obtained when this serum was tested with hordein in dilutions as low as 1–1,000, nor with the above-mentioned heterologous proteins. Here again no passive anaphylaxis could be obtained when 0.1 gm. hordein was injected into each of 5 guinea-pigs, 48 hrs. after they had received 3 c.c. of the serum intraperitoneally.

Experiment 9.—Hordein was injected into a rabbit intraperitoneally, as follows: 0.5 gm., February 17; 0.6 gm., February 21; 0.75 gm., February 25; 0.75 gm., February 28; 1 gm., March 2; 1 gm., March 7 ; 1 gm., March 10. Ten days later the animal was killed and the serum collected. It was found to give a complement fixation with hordein in 1–1,000,000 dilution and also to wheat and rye gliadin in the same dilution. With wheat proteose the reaction was negative, while with malt proteose it was positive in a 1–500,000 dilution. This antiserum also gave a precipitin reaction, as follows: with hordein in 1–100,000, with wheat gliadin 1–200,000, and with rye gliadin 1–40,000, but with wheat and malt proteose the reaction was negative. The passive anaphylactic reaction was also positive, a slight reaction being obtained with hordein and wheat gliadin, and a moderate reaction with rye gliadin.

It will be noted that the results obtained in Experiment 9 are decidedly different from those of the eight preceding experiments, in that the precipitin and passive anaphylactic reactions, previously not demonstrable, were now definitely positive, and in that

at the same time the apparent marked specificity shown in the earlier experiments had been so altered that the closely related protein gliadin reacts with the antihordein serum. (We are unable to explain why a stronger reaction was obtained with wheat gliadin than with hordein, and suspect some undetected error.)

Experiment 10.—This was a duplicate of the preceding experiment, the same doses being given at the same time, etc. It is of interest in that the animal's serum was of slightly lower titre, particularly as regards the precipitin test, and at the same time the passive anaphylactic reaction was positive only with hordein. In other words, this weaker serum showed more tendency to specificity, and a narrower range of reaction.

Inasmuch as gliadin and hordein differ from most other proteins in being soluble in alcohol, and are also characterized by yielding much glutaminic acid, proline, and ammonia, and very little arginine and histidine, and not more than traces of lysine, other vegetable proteins which yield much arginine were tried for comparison. Edestin and squash-globulin were selected because they dissolve easily, and also because our previous anaphylactic experiments showed the former to be relatively inactive while the latter was extremely toxic to sensitized animals when injected intraperitoneally.

Experiment 11.—Two rabbits were immunized to edestin from hemp-seed, by intraperitoneal injections as follows: January 17, 0.3 gm.; January 21, 0.4 gm.; January 24, 0.4 gm.; January 27, 0.5 gm.; January 31, 0.5 gm.; February 3, 0.5 gm.; February 7, 0.5 gm. On February 28 the surviving animal was killed and the serum collected (the other had died after the fourth injection). The antiserum gave with edestin positive complement fixation and precipitin reactions, each in 1–100,000 dilution, as well as a very severe passive anaphylactic reaction. The precipitin reaction was also given with the closely related flax-seed globulin, in a 1–10,000 dilution, while the complement fixation was here negative. This is of interest in view of the observations of White and Avery, that 2 guinea-pigs sensitized with edestin reacted typically to flax-seed globulin, one fatally.

Experiment 12.—Six doses, containing a total of 2.8 gm. of squash-seed globulin, were injected intraperitoneally, at intervals of 3 or 4 days, into 2 rabbits, and 10 days after the last injection they were killed. Their serum then gave about an equal titre on a preliminary complement fixation test, which was positive, in 1–100,000 dilution; it also gave the precipitin test when squash-seed globulin was used as antigen. The passive anaphylactic reaction gave striking results, being severe with squash-seed globulin. When the chemically similar, but genetically distantly related excelsin from the Brazil-nut, *Bertholletia excelsa*, was used as antigen, the complement fixation reaction was positive only in 1–1,000, and the precipitin reaction in 1–10,000 dilution.

These results are in striking agreement with those obtained by anaphylaxis tests;[1] that is, of 11 guinea-pigs sensitized with squash-seed globulin, 7 reacted, 1 even fatally, when excelsin was subsequently injected. This forms a striking illustration of the fact emphasized in our previous articles, that specificity almost certainly depends upon chemical rather than on biological relationships of the proteins used.

The results of the last two experiments indicate that when the precipitin reaction is given with very dilute solutions, the serum will also readily produce passive anaphylaxis. The control experiment (No. 13) with egg-white also shows this. At the same time hordein immunization, which at first shows only the complement fixation test, when carried farther leads to the appearance of the precipitin and passive anaphylaxis reactions.

Experiment 13.—Control with egg-white. To control these experiments a rabbit was given, intraperitoneally, 57.5 c.c. of egg-white in 6 doses during 24 days, and 12 days after the last injection it was bled to death. A sample of serum drawn on the fifteenth day gave no precipitin reaction with egg-white in dilutions from 1–10 to 1–10,000. On the twenty-third day a positive precipitin reaction was given by dilutions of 1–10,000 and on the thirty-sixth day in dilutions of 1–200,000, while positive complement fixation was then obtained in dilutions of 1–10,000,000, the greatest dilution tried. Passive anaphylaxis was conferred upon guinea-pigs by intraperitoneal injection of doses of from 0.5 to 3.0 c.c. of the 36-day serum, all reacting severely and about alike when given 1 c.c. of egg-white intraperitoneally 48 hrs. later.

Experiment 14.—Control with human ascites fluid. A total of 52.5 c.c. of human ascites fluid was injected intravenously into a rabbit in 6 doses during 15 days, and 10 days after the last injection the animal was bled to death. This serum then gave a positive complement fixation reaction with ascites fluid in dilutions up to 1–1,000,000, and a precipitin reaction with dilutions up to 1–100,000. Passive anaphylaxis experiments were not very successful, for 8 guinea-pigs which had received injections of 0.5 to 3.0 c.c. of the serum showed only very slight symptoms when 1 c.c. ascites fluid was given 48 hrs. later.

The results of these experiments with vegetable proteins are summarized in Table 1, p. 372.

DISCUSSION OF RESULTS.

Summarizing these experiments, as detailed in the protocols and Table 1, we have the following observations:

Three rabbits immunized with wheat gliadin yielded a serum which gave a specific complement fixation reaction with wheat gliadin even in dilutions up to 1–100,000, but not with rye gliadin or hordein in dilutions of 1–2,000. These sera failed to

[1] *Jour. Infect. Dis.*, 1911, 8, p. 66.

TABLE 1.

Immunization		Complement Fixation			Precipitin Reaction		Passive Anaphylaxis	
Material	Route	Hordein	Gliadin Wheat	Gliadin Rye	Hordein	Gliadin Wheat	Hordein	Gliadin Wheat
Gliadin wheat—								
(1) 1.2 gm....	Vein	.....	.....	.....	.....	Negative	.....	.....
(2) 2.0 gm....	"	.....	Positive up to 1:4,000	.....	.....	Negative	.....	Negative
(3) 4.7 gm....	Peritoneum	Negative at 1:2,000	Positive up to 1:10,000	Negative at 1:2,000	.....	.....	.....	.....
(4) 4.7 gm....	"	Negative at 1:2,000; also negative to malt and wheat "proteoses"	Positive up to 1:100,000	Negative at 1:2,000	.....	Negative	.....	Negative
Hordein—								
(5) 2.6 gm....	Vein	Positive up to 1:400,000	Negative at 1:2,000	Negative at 1:2,000	Negative at 1:1,000	.....	Negative	.....
(6) 2.6 gm....	"	Positive up to 1:600,000	Negative at 1:2,000	Negative at 1:2,000	Negative at 1:1,000	.....	Negative	.....
(7) 3.5 gm....	"	Positive up to 1:200,000	Negative at 1:2,000	Negative at 1:2,000	.....	.....	.....	.....
(8) 3.5 gm....	"	Positive up to 1:300,000; negative to malt "proteose"	Negative at 1:2,000; also negative to wheat "proteose"	Negative at 1:2,000	Negative at 1:1,000	Negative at 1:2,000; also negative to rye gliadin	Negative	.....
(9) 5.85 gm...	Peritoneum "	Positive up to 1:1,000,000; also positive to malt "proteose" 1:500,000	Positive up to 1:1,000,000; negative to wheat "proteose" 1:2,000	Positive up to 1:1,000,000	Positive at 1:100,000; negative to malt and wheat "proteose"	Positive at 1:200,000 and to rye gliadin 1:40,000	Slight reactions	Slight. With rye gliadin moderate reactions
(10) 5.8 gm...	"	Positive up to 1:500,000; also positive to malt "proteose" 1:500,000	Positive up to 1:1,000,000; also positive to wheat "proteose" 1:500,000	Positive up to 1:500,000	Positive at 1:40,000; negative to malt and wheat "proteose"	Positive at 1:4,000 to both wheat and rye gliadin	Slight reactions	Doubtful with both wheat and rye gliadin
Edestin—								
(11) 3.1 gm...	Peritoneum	Positive with edestin at 1:50,000 " " flax globulin at 1:10,000			Positive with edestin at 1:100,000 " " flax globulin at 1:10,000		Severe with edestin	
Squash globulin—								
(12) 2.8 gm...	"	Positive with squash globulin at 1:100,000 " " excelsin at 1:1,000			Positive with squash globulin at 1:100,000 Positive with excelsin at 1:10,000		Severe with squash globulin	

give positive precipitin reactions with wheat gliadin in 1–2,000 dilution, which is as strong a solution as usually can be used on account of the precipitate which often forms on mixing more concentrated solutions of gliadin with serum. The usual doses (0.5–3.5 c.c.) of these antisera did not render guinea-pigs passively anaphylactic to gliadin.

Since the preparations of hordein dissolved more easily than those of gliadin, we made most of our other experiments with this representative of the alcohol-soluble vegetable proteins. Three different sets of rabbits were immunized to hordein, one in the spring of 1912, one in the summer of 1912, and one in the winter of 1913. Each of these three sets received about the same number of injections during the same length of time, the first two intravenously, the last one intraperitoneally. The conditions under which these experiments were performed differed in these respects: (*a*) the time of year, (*b*) amount of material injected, and (*c*) possibly in the breed of rabbits. The first set, which received 2.63 gm. of hordein each, yielded an antiserum of high titre, as shown by the complement fixation reaction in dilutions of 1–400,000 to 1–600,000, specific in that it reacted only to hordein, and failed to react to the closely allied gliadin from wheat or rye. The precipitin and passive anaphylactic tests were negative with hordein.

The second set, receiving 3.5 gm. each, produced an antiserum which reacted similarly except that possibly there was a slight complement fixation reaction with wheat gliadin and malt proteose in 1:2,000 dilution.

Set 3, receiving 5.6 gm. each, showed an interesting change in the character of the resulting antiserum. In this case the antiserum for hordein gave the complement fixation reaction in relatively high dilution with the closely related heterologous proteins from wheat and rye and proteose from malt, but not with proteose from wheat. With the precipitin test, some degree of quantitative specificity was shown, the reaction being exhibited in highest dilution with hordein, in lower with wheat gliadin, in lowest with rye gliadin. Antiserum from one animal when injected into guinea-pigs sensitized them to hordein and to rye and wheat gliadin (passive anaphylaxis). Antiserum from another animal gave this passive

sensitization only for hordein. This latter serum also showed a similar definite gradation in the precipitin reaction; in both this and the complement fixation tests it had a lower titre than the first serum.

One rabbit was immunized to edestin from hemp-seed and another to the globulin from squash-seed. Since these proteins often cause death when injected intravenously,[1] we used intraperitoneal injections for the following experiments and thereby avoided all untoward symptoms. About 3 gm. of each of these two chemically similar proteins were given to each animal.

The antiserum obtained after immunizing with the globulin from the squash-seed gave positive reactions with the complement fixation, the precipitin, and the passive anaphylaxis tests. With the complement fixation test this serum reacted in dilutions not greater than 1:100,000, thus showing a much lower activity than the hordein antiserum which reacted in dilutions of 1:1,000,000; it, however, gave a severe passive anaphylactic reaction, whereas hordein antiserum gave only a mild one. It is interesting to note in this connection that the antiserum produced by the globulin of the squash-seed reacted with the excelsin from the Brazil-nut. This latter protein is similar to the globulin of the squash-seed, both in its physical properties and in the proportion of amino-acids yielded by hydrolyzing with strong acids. Here again we have another indication that these biological reactions are determined by the chemical constitution of the proteins.

Edestin immunization produced an antiserum which gave the complement fixation reaction in dilutions up to 1:50,000, the precipitin reaction at 1:100,000, and caused a strong passive sensitization.

The chief conclusions to be derived from these experiments are briefly as follows:

1. Carefully purified preparations of vegetable proteins readily produce antisera.

2. The antisera obtained in our experiments differed in their range of reactions, some giving only the complement fixation, some the complement fixation and precipitin tests, while others in addition conferred passive anaphylaxis to guinea-pigs.

[1] Autopsies show that the lung capillaries are occluded by granular matter (precipitated protein?).

3. Antisera to the same protein obtained from different individual animals differ in their reactions, for some unknown cause.

4. An antiserum at one stage of its development may be apparently of sharply limited specificity, giving only the complement fixation reaction with the homologous protein, while a sample taken later from the same animal, after the antibody content has increased, will react with heterologous proteins having similar chemical and physical properties.

5. At about the time the serum develops the complement fixation reaction with such heterologous proteins, the precipitin reaction as well as the passive anaphylaxis reactions appears, but at first may be limited to the homologous protein.

6. A specific complement fixation reaction in high dilution does not necessarily accompany reactions with the heterologous proteins, nor can such serum always produce the passive anaphylaxis reaction.

7. Both the precipitin and passive anaphylaxis reactions appear later in immunization than the complement fixation reaction, and seem to be closely related to each other in delicacy.

With these points in mind, it might be well to notice the relations of these reactions to those obtained by anaphylaxis alone, as described in our previous papers.

The first experiments, in which an antiserum was obtained that would react only with the homologous protein, seemed to show that the complement fixation reaction either is a more delicate reaction than anaphylaxis (which, with the same materials, gives reactions with related heterologous proteins), or that it is due to a different antibody. The later experiments show that when the animal is further immunized, positive complement fixation reactions can be obtained with closely related heterologous proteins, thus agreeing with the results of anaphylaxis experiments. At the time the precipitin reaction appears, the passive anaphylactic condition usually can be induced in guinea-pigs injected with this precipitating serum.

The important but insufficiently considered observations of Magnus[1] are fully confirmed by our present experiments with *pure proteins*. Magnus used extracts of plant tissues and found by

[1] *Ber deut. Bot. Gesellsch.*, 1908, *26a*, p. 532.

carefully conducted precipitin tests that *the degree of immunization determines the range of reaction.* For example, when an animal is immunized but a short time with extracts of the seeds of one of the cereals, it yields a serum which precipitates only the extract of the same species; later in the course of immunization, precipitins appear for extracts of closely related species, and progressively a wider and wider list of cereals reacts, until finally precipitates are obtained with extracts of all the *Gramineae.* Nevertheless, even with this extreme degree of immunity the serum gave no reaction with extracts derived from plants not belonging to the *Gramineae.* As the order of appearance of reactions with heterologous extracts was always the same, Magnus holds that it is possible in this way to secure a standard for estimating the relationship of plants by biochemical means.

As to whether or not the antibody responsible for fixation of the complement is identical with that which causes the precipitin reaction, the anaphylactic phenomena, etc., we are unable at present to state, but inasmuch as the antisera which gave the precipitin test also caused passive anaphylaxis, it is possible that one and the same antibody is common to these two reactions. On the other hand, a larger amount of a common antibody may be required to produce these reactions than is required for the complement fixation reaction.

The questions raised by these experiments deserve further study and the accumulation of much more data before definite conclusions can be drawn. Unfortunately, our present knowledge of the actual chemical relations of different individual proteins to one another is too meager to serve as a guide in interpreting the results of such experiments, but it is not impossible that chemical relationships may be indicated by these biological reactions, which in conjunction with further chemical studies may ultimately lead to a better knowledge, not only of the nature of the processes causing these mysterious changes in the serum, but also of the chemical constitution of the proteins.

THE ANAPHYLACTOGENIC ACTIVITY OF SOME VEGETABLE PROTEINS.*†

THE BIOLOGICAL REACTIONS OF THE VEGETABLE PROTEINS. V.

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In an earlier paper[1] we gave the results of anaphylaxis experiments in which carefully purified preparations of a large number of typical vegetable proteins were used. These preparations represent the principal part of the reserve protein of the seeds from which each had been obtained, and differ in their solubility and chemical constitution to a marked extent from the protein constituents of the sera and other fluids of animal origin, with which most anaphylaxis experiments had previously been made. In the course of our investigation it was found that while the minimum sensitizing dose of edestin from the hemp-seed, *Cannabis sativa*, was practically the same as that of the globulin from the squash-seed, *Cucurbita maxima*, and likewise that the minimum quantities of these proteins required to produce anaphylactic intoxication were also about the same, great differences in activity existed between intoxicating doses which were larger than the minimum. Thus, of 19 sensitized guinea-pigs injected with 100-mg. doses of squash-seed globulin 18 died of anaphylactic shock, whereas in 15 similar experiments with edestin but 2 fatal reactions were obtained.

This fact led us to suggest that the difference might be due to differences in the solubility of these proteins in the body fluids. This possibility was supported by the fact that, in the peritoneal cavity, edestin is precipitated from the dilute alkalin solutions injected, and consequently may not be redissolved and carried to the blood quickly enough to cause severe intoxication, where, according to White and Avery,[2] 0.5 mg. edestin gives fatal reactions.

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† A part of the expense of this investigation was shared by the Carnegie Institution of Washington.

[1] *Jour. Infect. Dis.*, 1911, 8, p. 106.

[2] *Ibid.*, 1913, 13, p. 103.

If such were the case the severity of the symptoms would be limited by the solubility of the protein in the peritoneal fluids rather than by the amount injected. None of the vegetable proteins which we then used possessed the high solubility in dilute saline solutions which characterized the chief protein constituents of such animal fluids as had previously been used in anaphylaxis experiments, and which had been shown to produce anaphylactic intoxication in much smaller doses.

We have now made a series of experiments under uniform conditions in order to obtain more extensive data concerning the relative anaphylactic toxicity of several of these reserve proteins of seeds, and have extended their scope to include preparations of the so-called *proteoses* from the Brazil-nut, *Bertholletia excelsa*, and the Soy-bean, *Glycine soja*. These preparations represent the part of the protein which is not precipitated from the sodium chlorid extract of this seed after prolonged dialyzing and subsequently heating the filtered solution to 100° C. They were separated from their concentrated aqueous solutions by adding alcohol, and purified by reprecipitating. These preparations contain all of the protein remaining in the extract after separating the globulins and albumins, and may consist of one or several individual protein substances. Nothing definite is known as to the chemical constitution of these proteoses, nor their relations to proteoses obtained by the action of proteolytic enzymes. They are called proteoses simply because they resemble this class of proteins in solubility. Whether they exist preformed in the seed, or are formed from the reserve protein of the seed by the action of the seed enzymes during the process of extraction, etc., is not known. (The biological reactions of the vegetable "proteoses" will be discussed in a forthcoming paper.)

The important point in connection with these present experiments is simply that these preparations from the Brazil-nut and Soy-bean represent a type of vegetable protein which is highly soluble in water and is precipitated from its solutions with great difficulty.

The results of our new experiments are given in Table 1. The terms used in describing the severity of the reactions are applied

TABLE 1.

MINIMUM INTOXICATING DOSE OF SOME VEGETABLE PROTEINS WHEN INJECTED INTRAPERITONEALLY INTO GUINEA-PIGS SENSITIZED 3 WEEKS PREVIOUSLY WITH 1 MG. OF THE SAME PROTEIN.

	0.05 mg.	0.1 mg.	0.5 mg.	1 mg.	2 mg.	3 mg.	5 mg.	10 mg.	20 mg.	40 mg.
1. Castor-bean globulin (Ricinus communis)	.....	.....	Slight	Doubtful Moderate	Sligbt	Slight Doubtful Moderate	Sligbt	Severe	.....	.....
2. Squasb-seed globulin (Cucurbita maxima)	.....	.....	o	Slight Doubtful	.....	Sligbt	Moderate Moderate	Severe	Severe	.....
3. Edestin (Cannabis sativa)	.....	.....	o	Doubtful	Slight	.....	Slight	Moderate	Severe	Severe
4. Excelsin (Bertholletia excelsa)	.....	.....	o	Doubtful Sligbt	Moderate	.....	Moderate	Moderate	Fatal	.....
5. Pea legumin (Pisum sativum)	.....	.....	o	Slight o	Doubtful o	....	Slight Moderate	Slight Doubtful Doubtful	Moderate	.....
6. Vicilin (Pisum sativum)	.....	.....	o	Doubtful Doubtful Severe	Sligbt	.....	Slight Severe Doubtful	Sligbt	Slight	Moderate
7. Vignin (Vigna sinensis)	.....	.....	o	Slight	Moderate	.....	Severe Moderate	Fatal Fatal	Fatal	.....
8. Glycinin (Soja hispida)	.....	.....	Doubtful	Slight	Slight	.....	Sligbt	Moderate	Severe Slight	.....
9. Wbeat gliadin (Triticum vulgare)	.....	.....	Doubtful o Severe	Slight Moderate	Severe	.....	Fatal	Severe Doubtful	.....	.....
10. Hordein (Hordeum vulgare)	.....	.....	Slight Moderate	Slight Moderate	Slight Moderate	Moderate	Moderate Fatal	Severe Severe	.....	.....
11. Zein (Zea mays)	.....	.....	o	Slight Doubtful	Sligbt	.....	Moderate	Moderate	.....	.....
12. Brazil-nut proteose (Bertholletia excelsa)	Sligbt	Severe	Fatal 68 min. Fatal 80 min.	Fatal 40 min.	Fatal 38 min.	.....	.....	.....	.....	.....
13. Soy-bean proteose (Glycine soja)	Moderate Doubtful with 0.01 mg.	Moderate Moderate	Moderate	Severe	Fatal 2 hrs.	.....	.....	.....	.....	.....

with the same significance as in all our previous articles, and are defined in the first paper.[1]

The eight proteins appearing first in this table all belong to the group of globulins; that is, they are insoluble in neutral aqueous solutions but soluble in neutral saline solutions. None of them, except vicilin, dissolves to any marked extent in such dilute saline solutions as do the globulins present in most animal fluids. They are, therefore, probably absorbed from the peritoneal cavity relatively slowly.

Gliadin and hordein represent a totally different type of solubility, being practically insoluble in dilute neutral saline solutions and but slightly soluble in pure water. Their most marked characteristic is their solubility in alcohol of 80–90 per cent. Zein belongs to the same class, but is insoluble in water or saline solutions and very readily soluble in alcohol of 90–95 per cent.

The solubility of the proteoses has already been described.

It is difficult to interpret accurately the results given in the table according to the relative solubility of these proteins, for with the exception of the proteoses, the degree of solubility of each when injected into the peritoneum will depend on factors of which at present we have but little knowledge. Thus while edestin is insoluble in pure water it is readily dissolved by water containing minute amounts of acids or alkalies. The addition of a very small quantity of a neutral inorganic salt at once precipitates the salt of edestin formed by its union with the added acid or base, while a larger quantity of the same neutral salt will redissolve the precipitate. The proportion of inorganic salt thus causing precipitation, or resolution, depends on the proportion of acid or alkali combined with the edestin. In view of such intricate solubility relations it is evident that with our present knowledge it is impossible to draw definite conclusions respecting the amount of injected protein which may be precipitated in the peritoneal cavity.

The results given in the table, however, are in the main in harmony with what is at present known concerning the relative ease with which the protein might be expected to be precipitated from the dilute alkalin solutions used for intoxication. Thus,

[1] *Loc. cit.*

with the exception of edestin and vicilin, toxic symptoms begin to appear when one milligram of the globulin is used, while with larger quantities the results obtained with these several globulins do not differ from one another very greatly. Edestin is the least toxic of this series, and from such comparisons of its solubility with that of the other globulins, as have yet been made, it seems probable that it is more easily precipitated in the peritoneal cavity than they are. Vicilin appears to be the most toxic of this series of globulins, which is in harmony with the fact that it dissolves abundantly in much more dilute saline solutions than do any of these other globulins.

The alcohol-soluble proteins, gliadin and hordein, are about equally toxic, and more so than any of the other proteins in this list except the proteoses. Whether this is due to their peculiar chemical constitution, or to their more ready solubility in the body fluids, cannot be definitely determined from available data. That the latter is the more probable explanation is indicated by the fact that zein, which is much more easily precipitated from dilute alkalin solutions than are gliadin or hordein, is distinctly less toxic anaphylactically. The strongest evidence that the degree of toxicity is determined by the solubility of the injected protein is shown by the experiments with the proteoses from the Soy-bean and the Brazil-nut, for the minimal lethal dose of these preparations is far smaller than that of any of the other vegetable proteins tested, and is of the same order of magnitude as that of egg albumin or of serum proteins.

It is impossible to reproduce accurately the conditions that obtain within the animal body, and thus determine the exact degree of solubility of our vegetable proteins in either the peritoneal fluids or the blood. The best we can do is to determine their precipitability by serum, and experiments of this sort indicate that there is a very close correspondence between the precipitability of the vegetable proteins and their toxicity for sensitized animals. Such experiments were conducted as follows:

To small test tubes, each containing 0.5 c.c. of normal beef serum, kept sterile for some time with chloroform, which was removed before using, was carefully added 0.1 c.c. of the solution of the protein to be tested, dissolved in 0.1 per cent NaOH,

which was the solvent usually employed in our animal experiments. The solution was run upon the surface of the serum with a minimum of admixture, and the tubes stoppered. After the solution had stood for one hour at room temperature the amount of turbidity or precipitate at the line of contact was noted and indicated in the table as "zone," the standard for comparison being a set of tubes prepared at the same time, and in the same proportions, with edestin. The tubes were then thoroughly shaken and left in the icebox for 18 hrs., when the amount of sediment was again observed and compared with that in the tube to which edestin had been added, being indicated in the table as "precipitate." Since the serum was always more or less opalescent or turbid, as were also many of the protein solutions, the reactions given in the following table as "traces" or "doubtful" have little or no significance.

TABLE 2.

PRECIPITATION OF VEGETABLE PROTEINS BY SERUM.

Strength of Protein Solution	1 Per Cent	0.2 Per Cent	0.1 Per Cent	0.05 Per Cent	0.01 Per Cent	0.005 Per Cent	Percentage of Fatal or Severe Reactions with 100 mg. Doses
Edestin: zone	+++++	+++	++	++	+	+	33
precipitate	+++++	++	+	+	Tr.	o	
Zein: zone	++++	++	+	Tr.	d	d	30
precipitate	+++++	+	Tr.	Tr.	o	o	
Gliadin (wheat): zone	+++	++	+	Tr.	d	o	67
precipitate	++	+	Tr.	o	o	o	
Hordein: zone	+++	++	+	Tr.	d	d	69
precipitate	++	+	+	Tr.	o	o	
Excelsin: zone	++	+	+	+	Tr.	Tr.	94
precipitate	+++	o	o	o	o	o	
Squash-seed globulin: zone	++	Tr.	Tr.	o	o	o	95
precipitate	+++	Tr.	o	d	d	o	
Flax-seed globulin: zone	+	+	Tr.	d	d	o	.....
precipitate	+	o	o	o	o	o	
Vignin: zone	Tr.	o	o	o	o	o	100
precipitate	+	o	o	o	o	o	
Proteose, excelsin: zone	Tr.	o	o	o	o	o	100
precipitate	d	o	d	o	o	o	
Proteose, Soy-bean: zone	Tr.	o	o	o	o	o	100
precipitate	o	o	o	o	o	o	

Arranging the preparations in the apparent order of precipitation, we find that edestin and zein, which least often produce severe or fatal intoxications on intraperitoneal injection into sensitized guinea-pigs, give by far the greatest amount of precipitate with serum. At the other end of the list come the vegetable "proteoses," such as Brazil-nut proteose and Soy-bean proteose, which we have found to be quite as toxic (anaphylactically) as serum or egg-white proteins, which likewise give little, if any, precipitate with beef serum under the conditions of the experiment. Flax-seed globulin, squash-seed globulin, vignin, and excelsin,

which are somewhat less active, produce more precipitate than the "proteoses," but less than hordein and gliadin which they exceed in anaphylactic toxicity. Doses of 50–100 mg. of gliadin or hordein give fatal or severe reactions with sensitized guinea-pigs less frequently than do flax-seed globulin, squash-seed globulin, excelsin, or vignin. Thus, in an earlier series of experiments, the percentage of fatal and severe reactions with 100-mg. doses of these proteins was as follows: squash-seed globulin, 95; vignin, 100; excelsin, 94; hordein, 69; gliadin, 67; zein, 33; edestin, 30. The percentage of fatal reactions was: squash-seed globulin, 95; vignin, 72; excelsin, 67; hordein, 44; gliadin, 17; edestin, 13; zein, 0.

On the other hand, in the series of experiments recorded in this paper, 5 mg. of gliadin or hordein, 10 mg. of vignin, and 20 mg. of excelsin were the smallest quantities, respectively, causing death. With the other proteins even in doses of 20 mg. no fatal reactions were obtained. We thus see that the minimum intoxicating doses of these proteins bear no exact relation to their relative toxicity when given in larger doses; but it is of significance that the, comparatively speaking, extremely soluble "proteoses" are toxic in the smallest doses and also almost always produce fatal reactions in sensitized animals.

The efficiency of these vegetable proteins in producing severe intoxication in sensitized animals, therefore, corresponds in a general way to their precipitability by serum as thus determined, and presumably depends upon the extent to which they are dissolved and absorbed into the blood after injection into the peritoneal cavity.

CONCLUSIONS.

The data presented in this paper support the assumption that the severity of the anaphylaxis reactions produced by intraperitoneal injections of dilute, alkalin solutions of vegetable proteins is, approximately, in inverse ratio to their relative precipitability when their solutions are mixed with the peritoneal fluid.

Proteins like edestin, which are readily precipitated, and only slowly redissolved, under conditions similar to those presumably prevailing in the peritoneum, rarely produce a fatal intoxication;

whereas those that are less easily precipitated, and more readily dissolved, give fatal reactions in much smaller doses.

The lethal dose of those vegetable proteins which are readily soluble in pure water, or in very dilute saline solutions, is smaller than that of those which are soluble only in comparatively strong saline solutions.

The minimum intoxicating dose of most of the vegetable globulins (i.e., proteins insoluble in pure water but soluble in saline solutions) administered intraperitoneally is from 1 to 2 mg., but from 5 to 10 mg. are usually required for severe intoxication.

On the other hand, the minimum intoxicating dose of those so-called vegetable proteoses which are readily soluble in pure water and are uncoagulable by heat is much smaller, moderate to severe reactions being obtained with 0.05–0.1 mg. and fatal results with 0.5–2 mg.

THE IMMUNOLOGICAL REACTIONS OF THE PROTEINS OF THE HUMAN PLACENTA WITH SPECIAL REFERENCE TO THE PRODUCTION OF A THERAPEUTIC SERUM FOR MALIGNANT CHORION-EPITHELIOMA.*

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Various authors have reported the production of immune sera, which they believed to be specific for certain cells or cell complexes. The work to be reported in this article was undertaken to determine whether antisera specific for human placenta, or for certain of its proteins, could be obtained. The placenta, especially the fetal portion, is known to behave in many respects as a tissue foreign to the mother, and therefore seemed to offer a more favorable field for such work than the permanent and essential tissues of the body. It was hoped that a specific antiserum to placental tissue, if it could be produced, would be of particular value in the treatment of chorion-epithelioma, either alone or as an adjunct to surgical procedure. Considerable work has already been reported concerning the production of specific cytolytic antisera by the injection of nucleoproteins of organs, which in some cases have been stated to have a therapeutic value. Beebe has been the chief exponent of this work, which, however, has not been confirmed by the work of Pearce and others.[1] In view of these facts, this work, then, seemed desirable (1) as a repetition of the investigation of immune reactions of the nucleoproteins (which can hardly be considered as definitely settled); (2) because little work has been done with the proteins of the placenta, the nucleoprotein being the only one so far used; and (3) because the special phase of this problem, the application of antiplacental sera to the therapy of chorion-epithelioma, had not been considered.

The literature on the subject is not very extensive. So far as the placenta itself is concerned, we have an excellent review by

* Received for publication February 2, 1914.

[1] See review by Wells, *Ztschr. f. Immunitätsf.*, 1913, 19, p. 599.

Frank[1] in 1907, covering the subject to that time. I can do no better than give a brief summary of Frank's paper.

Halban had accumulated numerous clinical observations, from which he adduced that the growth of the placenta causes a hypertrophy and hyperemia of the uterus and neighboring organs. The only experimental work in support of this was that of Lane-Claypon and Starling, who found that rabbit placenta injected into virgin rabbits produces hypertrophy of the breasts. Kollman had observed that the fetal syncytium underwent lysis in the maternal fluids. Scholten and Veit tried to prove this experimentally by immunizing rabbits with human placenta; they reported producing an antiserum which dissolved placental cells *in vitro*. Leipman was unable to confirm this work. He found, however, that such an antiserum contained precipitins for placenta and placental blood, but admitted that the washed placenta contained general body proteins as well as special placental cell proteins, and that absorption of the "general" proteins was first necessary to differentiate them from the specific placental proteins. Weichardt, Opitz, and Wormser, in attempting to repeat Leipman's work, obtained negative results; while Kawasoye and Freund report results similar to those of Leipman. Frank's work, which appears thorough and adequately controlled, showed that injections of rabbit placenta into rabbits produced no antibodies, as shown by the precipitin test. Injection of human placental "nucleoprotein," with a content of 0.35 per cent phosphorus, prepared as nearly as possible blood-free, did not produce any antibodies that could be detected by the precipitin and complement fixation tests. Immunization with suspensions of blood-free placental cells, however, did produce antisera as shown by the precipitin, complement fixation, agglutination, and hemolytic tests, but at no time did the antisera show any demonstrable cytolytic action. None of these reactions, however, was specific for placental cells, and they are referred to by Frank as weak "human reactions." In his conclusions he states that "nucleoproteins act merely as mild toxic agents, without specific qualities."

Since the appearance of this work in 1907 several articles have been published which have a bearing on the subject, especially as regards the individuality of the fetus in relation to the mother. Zuntz[2] has shown this by the specific character of the enzymes of the fetus and placenta. He mentions the work of other authors showing that there is a difference in the agglutinative power of maternal and fetal serum. The work of Abderhalden on specific enzymes for placental proteins is too well known to need more than mention.

Anderson and Rosenau[3] have reported the sensitization of guinea-pigs to guinea-pig placental extract, with pronounced anaphylactic symptoms resulting when the intoxicating dose of the same material was given, indicating that the placenta is biologically foreign to animals of the same species. They suggested that in some cases eclampsia might be explained on this basis. Thies[4] and Gräfenburg,[5] after an extensive

[1] *Jour. Exper. Med.*, 1907, 9, p. 263.
[2] *Ergeb. d. Physiol.*, 1908, 7, p. 403.
[3] *Jour. Med. Research*, 1908, 14, p. 37.
[4] *Arch. f. Gynäk.*, 1910, 92, p. 513.
[5] *Ztschr. f. Geburtsh. u. Gynäk.*, 1911, 69, p. 270.

series of experiments, held that during pregnancy the mother is sensitized by small amounts of fetal protein and that if a considerable quantity of fetal blood is at one time introduced into the mother anaphylactic symptoms follow. Johnstone[1] and Fellander[2] were unable to confirm these results and Williams, Lynch, and other obstetricians do not believe that this is the true explanation of eclampsia.

These experiments, while not in harmony, at least suggest the biochemical individuality of the fetus and placenta, the bearing of which is at once evident on our problem, as it would indicate that if the placental material could be freed from other tissues and blood, specific antisera might be produced of value in the treatment of eclampsia and of malignant chorion-epithelioma. Risel[3] and others have shown that spontaneous healing of the chorion-epithelioma occurs in a certain percentage of cases. The fact that this healing tendency is never seen to an equal degree with other malignant tumors is highly suggestive that placental tumor cells act as a foreign substance, to which the host may react with considerable success by means of antibody formation. Removal of the primary tumor is also said to be followed often by the healing of distant metastases. Hence the malignant placental tumors would seem to offer a better prospect for treatment by antisera than other forms of malignant tumors, which arise from essential tissue cells and which do not show so marked a tendency to spontaneous retrogression or healing.

Stimulus to work along the line of the production of specific cytotoxic sera in recent years is perhaps attributable more to the work of Beebe than to any other investigator. He used other tissues than placenta, but the general principles involved are the same. Beebe[4] found that animals injected with the "nucleoproteins" of various organs yielded an antiserum of high specificity, e.g., rabbits immunized to dog liver nucleoprotein produced an antiserum reacting specifically with this protein, as shown by the precipitin and agglutination tests. Injection of this serum into dogs produced characteristic lesions in the liver. Proteins from the thymus, kidney, pancreas, and other organs were also used.

[1] *Jour. Obst. and Gynec. Brit. Emp.*, 1911, 19, p. 253.

[2] *Ztschr. f. Geburtsh. u. Gynäk.*, 1911, 68, p. 26.

[3] *Ergeb. d. Allg. Path. u. Path. Anat.*, 1907, 11, Part 2, p. 928.

[4] *Jour. Exper. Med.*, 1905, 7, p. 733.

It should be mentioned that in a later communication[1] he says that it is not a simple matter to produce immune serum to nucleoproteins; e.g., of 5 rabbits only 1, and of 4 sheep only 1 produced active sera. As to specificity, he states that the serum has two factors, a general and a specific, and that to show the specific lesions in an animal receiving these sera, it must be killed at just the right time. Beebe[2] later extended his work and produced an immune serum for thyroid nucleoprotein, and used it in the treatment of goiter, obtaining results of some promise. Rodgers[3] reported a series of 10 cases treated in this manner, three of which were greatly benefited or cured. Taylor[4] made an antiserum for thyroid nucleoprotein according to Beebe's methods, but it did not give therapeutic results of value.

Bierry[5] reports that rabbits injected with nucleoprotein of dogs' kidney develop an antiserum, nephrotoxic to dogs. He states, however, that normal serum injected in the same amount produces some of these symptoms, tho not so marked. He does not state the effect of immunizing rabbits to dog serum and then trying this antiserum as a control.

Fiessinger[6] reported the production of an antiserum to liver and kidney nucleoprotein which showed some degree of relative specificity. Not all the work, however, gives such positive results. Pearce and Jackson,[7] working with the nucleoproteins prepared in a different manner (containing 1.74–2.1 per cent phosphorus), were unable to confirm Beebe's work. Beebe[8] criticized these authors in that in the preparation of their material it was brought to the boiling point, altho it has often been shown that proteins which are not coagulated and rendered insoluble are usually not altered in their antigenic properties by the amount of heating which these nucleoproteins received. Beebe again emphasizes the specificity of his products, stating that the precipitin and also the agglutination reactions were still obtained, tho in less degree, after the absorption of the general antibody content of the serum by treating it with dog muscle. He does not give complete protocols showing the dilutions in which these reactions occurred, nor whether they were always controlled with simultaneous tests on dog serum; nor does he mention treating dogs with serum of rabbits immunized to dog serum. He states that "the most searching and conclusive method, however, is by means of animal inoculations." It would seem, on the contrary, that if the active principle of the serum is an antibody, specificity could be more easily and certainly demonstrated by the precipitin and complement fixation reactions. The work of Nuttal, von Behring, Uhlenhuth, and others would lead us to that conclusion. Wells,[9] working with nucleoproteins prepared from dog liver by Beebe's method, found only a considerable toxicity, and no specific anaphylaxis reaction could be obtained. When

[1] *Brit. Med. Jour.*, 1906, 2, p. 1786.
[2] *Jour. Am. Med. Assn.*, 1906, 46, p. 484.
[3] *Ibid.*, 1906, 46, p. 487.
[4] *Ibid.*, 1911, 56, p. 263.
[5] *Compt. rend. Soc. de biol.*, 1903, 55, p. 476.
[6] *Jour. de physiol. exper.*, 1908, 10, p. 657.
[7] *Jour. Infect. Dis.*, 1906, 3, p. 742.
[8] *Jour. Am. Med. Assn.*, 1910, 55, p. 1212.
[9] *Jour. Infect. Dis.*, 1911, 9, p. 147.

guinea-pigs were given an intoxicating dose of dog serum after being previously sensitized with nucleoprotein, a moderate reaction was obtained. This held only when relatively large sensitizing doses of the nucleoprotein had been given. No reaction was obtained when only 1–2 mg. of nucleoprotein were given in the sensitizing dose. Finally, the most complete, as well as the most recent work, is that of Pearce, Karsner, and Eisenbrey.[1] Their materials, while not prepared exactly according to the method of Beebe, were open to none of the objections of the earlier work of Pearce and Jackson. The immune sera were tested by the precipitin, hemolytic, and agglutination reactions, none of which gave evidence of any specificity of the sera. The fractions, as tested by the anaphylactic method, showed a very slight relative organ specificity, but none for the different protein fractions of the same organ. Microscopic study of the organs of dogs receiving the various antisera gave no support to the view of specific action. They conclude that nucleoproteins play no important rôle in the production of cytolytic immune sera.

Doerr[2] gives an excellent review of the literature as regards specificity in anaphylaxis. He states that specificity of organs is difficult to demonstrate, as it is difficult to get organs free from serum. In case of tissues easily freed from serum, as the crystalline lens of the eye, the nails, etc., proteins may be prepared which show highly specific properties. These are the exceptions. The work of Wells and Osborne[3] shows that the question of specificity depends on the chemical composition of the proteins; that species specificity simply means that the material is really of demonstrably different chemical composition, and that where the composition is identical by the most delicate chemical methods, differing species specificity is not exhibited; and, on the other hand, that several chemically different proteins from the same source, animal or vegetable, may act as entirely distinct specific antigens. This is very well shown by the work with hen egg proteins.[4] Here Wells was able to demonstrate the presence in the hen's egg of at least 5 antigens distinguishable by anaphylactic methods, which corresponded to 5 different proteins distinguishable by chemical criteria, clearly demonstrating chemical specificity independent of species specificity. The applicability of these findings to this problem is evident. If we can get from a tissue a protein containing none of the general proteins of the serum, we may expect to get specific reactions for this tissue, but not otherwise.

Abderhalden and Kashiwado,[5] working with nucleoprotein of calves' thymus, by anaphylactic methods, reported a specific reaction. This conclusion was based on their inability to get a reaction when they gave an intoxicating dose of nucleoprotein from duck corpuscles. They conclude that apparently each kind of nucleus produces specific antibodies. Their protocols, however, fail to show whether or not their products were tested against serum of the animals furnishing them in order to exclude general reactions. The chief value of this work was in showing the non-antigenic character of the nucleic acids.

The production of immune serum with the bacterial nucleoproteins has received so much attention that it should be at least mentioned. Lustig[6] gives an excellent review of the literature as well as a considerable contribution to the subject. He

[1] *Jour. Exper. Med.*, 1911, 14, p. 44.

[2] Kolle and Wassermann, *Handbuch d. pathog. Microorg.*, 1913, 2, p. 947.

[3] *Jour. Infect. Dis.*, 1913, 12, p. 341. [4] *Ibid.*, 1911, 9, p. 147.

[5] *Ztschr. f. physiol. Chem.*, 1912, 81, p. 285.

[6] Kolle and Wassermann, *Handbuch d. pathog. Mikroorg.*, 1913, 2, p. 136.

concludes that nucleoproteins are important and chemically definite constituents of bacterial cells and bearers of antigenic functions.

This work with antisera prepared for bacteria seems to come the nearest to showing specificity of the nucleoproteins, but it must be remembered that here we are dealing with single cells in which the chief protein is believed to be a nucleoprotein, and therefore it is doubtful whether it has any advantages as to specificity over the whole cell. Levene's work[1] shows this very clearly. Furthermore, no record can be found of the testing of the antisera against nucleoproteins of bacteria with other proteins of the same bacteria. As a matter of fact the methods employed in the preparation of these so-called nucleoproteins of bacteria are so crude that it seems probable that the substances used under this name are really mixtures of many sorts of proteins.

Inasmuch as the antigenic value of the nucleoproteins has been so much emphasized it seems in keeping to give a brief discussion of these bodies and why they have been so extensively used, especially in the attempted production of antibodies to the complex cells of parenchymatous organs. Beebe appears to believe that some portion of these cells represents their specific properties. He suggests that as the nucleus is the controlling portion of the cell, it must contain this substance. It has commonly been held that the nucleus contains a high percentage of nucleoprotein, hence Beebe thinks that an antibody to this protein will be of highly specific character. This seems reasonable, and at once raises the question: What is a nucleoprotein? This is a difficult question to answer. Hammarsten says that "by nucleoproteins we designate those compound proteins which on cleavage yield protein and nucleic acid, occurring chiefly in nuclei, but widely distributed throughout the body in small amounts." Kossel considers them as proteins combined with a protesteric group, which contains phosphorus, and may be split off as nucleic acid on treatment with an alkali. The protein is a protamine and in some cases a histone, the latter being midway between the former and a protein. It is questioned whether the protamines are true proteins. Kossel considers them the simplest proteins, or as a nucleus of protein bodies. If this be true, then, it would seem that the antigenic properties would be small, for Wells[2] has shown that histone prepared from ripe cod testes possessed no antigenic properties, and Gay and Robertson[3] report that globin, which is a histone, after

[1] *Jour. Med. Research*, 1904, 12, p. 191.

[2] *Jour. Infect. Dis.*, 1911, 9, p. 147.

[3] *Jour. Exper. Med.*, 1913, 17, p. 535.

repeated injection into rabbits, failed to produce an antiserum as shown by the Bordet-Gengou fixation test. The other part, viz., the nucleic acid, which Hammarsten believes gives to the different nucleoproteins their characteristic properties, has been shown by Wells[1] and by Abderhalden and Kashiwado[2] to possess no antigenic properties as shown by the anaphylactic method. As opposed to this, it is of interest to note that Beebe states: "The fact that these bodies are so rich in nucleic acid leads one to believe that the production of antibodies may perhaps be caused as much by the acid portion of the compound as by the proteid." That nucleic acid should be an antigen is, indeed, highly improbable, in view of its relatively simple composition, for there is but little evidence that any substance except complete or nearly complete protein molecules have antigenic functions.[3]

Abderhalden[4] gives the composition of various nucleoproteins which have been studied by several authors. These show rather wide variations, particularly in the phosphorus content. This probably means difference in the amount of nucleic acid in combination with the protein, or in other words, the proportion and kind of protein present. There is no definite line between the nucleoproteins and the nucleins. With our present methods definite separation is probably impossible. Abderhalden[5] in discussing the matter says: "There is little wonder that the existence of the nucleoproteins should be repeatedly questioned." Among those who have made such criticisms may be mentioned Osborne and Harris.[6]

It is evident that different observers working with the nucleoproteins have in all probability been working with widely varying materials. In not a few instances the water-soluble extract has been acidulated and the precipitate, without further treatment, called nucleoprotein. In only a few instances has the material been analyzed. Even in the better preparations where the material is purified, we do not know how much it is changed each time some

[1] *Loc. cit.*

[2] *Loc. cit.*

[3] Wells, *Chemical Pathology* (Second Edition), 1914.

[4] *Handbuch d. Biochem. Arbeitsmeth.*, 1910, 2, p. 449.

[5] *Text Book of Physiological Chemistry*, p. 275.

[6] *Ztschr. f. physiol. Chem.*, 1902, 36, p. 132.

of the protein is split off, or that its character is changed. Wells[1] states: "It seems probable that the numerous chemical manipulations, especially the repeated solution in alkali and precipitation with acid, may be responsible for the inefficiency of these preparations of nucleoprotein and histone, for it is known that the action of acids and alkalies rapidly impairs the activity of proteins in respect to anaphylaxis as well as other biological reactions." As an instance of this he found[2] "that egg albumin converted into alkali albuminate, precipitated with acetic, washed and redissolved in alkali, caused in 0.1 gm. doses no symptoms in pigs sensitized to natural egg albumin." Acid albumin was still active, tho to a less degree than the natural albumin.

The difficulties mentioned would hold in working with simple cells. When we consider that with the placenta we have blood cells, serum, etc., intimately mixed with our placenta cells, the situation is the more complex. On the other hand, if an antiserum can be produced that is specific for placenta by immunizing with any one of the proteins that can be isolated from the placenta, it would seem probable that it might have therapeutic value not attainable with other antisera, for the reason that the placenta is a foreign tissue to the individual in which it is growing. Hence our antisera might be expected to be likely to act on this foreign tissue more exclusively, and to have less effect on the normal permanent tissues of the individual, than would an antiserum for the normal permanent body cells or their proteins.

EXPERIMENTS.

Preparation of materials.—Nucleoprotein of beef liver was first prepared, following Beebe's[3] method as closely as possible. The organs were washed free from blood, sliced, washed several times in running water, and ground to a pulp. This was extracted with two volumes of water in the cold, for 24 hrs., with occasional stirring, a little chloroform being added to prevent bacterial action. It was then strained through cheesecloth with slight pressure. The resulting extract was turbid and contained considerable material in suspension. An attempt was made to clear this by centrifugation, but it was impossible to get the "perfectly clear extract" described by Beebe. This may have been due in part to the fact that it was impossible to get a high enough speed with our machine. Acetic acid was then added to frank acid reaction, and the precipitate allowed to settle out over night, in the ice chest. The super-

[1] *Jour. Infect. Dis.*, 1911, 9, p. 147.

[2] *Ibid.*, 1909, 6, p. 513.

[3] *Jour. Exper. Med.*, 1905, 7, p. 733.

natant clear fluid was then pipetted off, and the remainder, containing the precipitate, filtered on a soft cone filter. This required about 5 hrs. The filtrate was fairly clear and showed only a faint turbidity on the addition of more acetic acid. The precipitate was then suspended in 0.85 per cent saline and washed 4 or 5 times by centrifugation. It was next dissolved in weak sodium carbonate solution, but it was impossible to get a perfectly clear solution. It was then reprecipitated with acid and filtered, this requiring a day or more. After the precipitate was removed it was washed 6–8 times with saline, but the wash-water was never quite clear. Each time it seemed that a little of the material was dissolved. Only a relatively small yield was finally obtained. This was dried over sulfuric acid.

Owing to the difficulties of this method, several modifications were made, eliminating the centrifuge to a large extent. Sheep and dog livers were used in order to perfect the method and to obtain other nucleoproteins for control. In washing the materials free from blood, after the first 2 or 3 rapid washings, a small amount of acetic was added in the hope that it would render the nucleoproteins less soluble. Instead of plain water for extraction of the nucleoproteins, N/20 to N/40 sodium carbonate was used, as the nucleoproteins are more soluble in this, and as, according to Hammarsten, they are insoluble in water. As it was impossible to get a clear extract by centrifugation, various methods of filtration were tried. Finally it was found that the best results were obtained by using a long-fiber asbestos on a Büchner suction filter, prepared in much the same way as an ordinary Gooch crucible. This gave a nearly clear filtrate, from which the protein was precipitated with acetic. When perfectly clear extracts were used the amount of precipitate obtained was decidedly less than when less care was taken to get clear filtrates. Usually the precipitate settles well, and in a short time, a few hours at most, the greater part of the fluid can be pipetted off. The remainder was removed by centrifuge and the precipitate redissolved in N/20 to N/40 sodium carbonate solution. It dissolved fairly well. It was filtered as before with considerable difficulty, again reprecipitated, allowed to settle a short time, filtered, washed several times, first with acidulated and finally with distilled water, which always dissolved some of the material. The precipitate was then removed and dried in a vaccum desiccator over sulfuric acid.

After trying out this method several times, fresh human placentas were obtained and treated in the same way. In all, 42 were used (which were furnished us by the Michael Reese Hospital through the kindness of Dr. J. W. Jobling). From 3 to 12 were worked up at a time. Some lots worked up more easily than others. In general, it was a slow, tedious process, and in some cases but a very small quantity of the nucleoprotein was obtained. In all, about 12 gm. of the thoroughly purified material were prepared.

In addition, other fractions of the placenta proteins were prepared as follows: the *globulin fraction*, by half saturation with $(NH_4)_2SO_4$ of the filtrate obtained after the first precipitation with acetic, purifying by redissolving and again half saturating twice, and removing the salt by dialysis. The *albumin fraction* was obtained by completely saturating the filtrate from the globulin with ammonium sulfate, dissolving the precipitate with the least

possible amount of water, resaturating at least twice, and removing the salt by dialysis.

Gelatin was obtained by treating the residue after the first extraction with weak carbonate to neutralize exactly, and boiling for 2 hrs., filtering and evaporating the hot filtrate to a small volume on the water bath. While still hot, 4 volumes of alcohol were added, which precipitated out the gelatin. After standing a few hours this can be easily filtered. The precipitate was dried a few hours, dissolved in hot water on the water bath, and filtered with considerable difficulty on a hot filter, thus removing all other proteins than gelatin. The gelatin was reprecipitated with alcohol as before, filtered out, and dried over sulfuric acid.

TABLE 1.

Material	Total Nitrogen Percentage	Purine Nitrogen Percentage	Phosphorus Percentage	Millons	Adam-Kewicz	Biuret	Xantho-proteic	Molisch
Nucleoprotein (A). Human placenta	13.35	.....	.....	+++	++	−+	++	o
Nucleoprotein (B). Human placenta	.....	.....	.....	+++	o	o	++	Trace
Nucleoprotein. Sheep liver	.....	.....	.....	++	Trace	±	+	Trace
Nucleoprotein (A). Cow's liver	.....	.....	.....	±	o	o	±	±
Nucleoprotein B^1. Cow's liver 1st ppt	11.76	0.24	0.19	++	+	++	++	o
Nucleoprotein B^2. Cow's liver 5th ppt	11.59	0.19	0.31	++	++	+	++	o
Nucleoprotein. Dog liver	13.56	Trace	0.77	.....	.....	.....	.....	.....
Globulin. Human placenta	15.4	o	o	.....	.....	.....	.....	.....
Albumin. Human placenta	14.56	o	Trace	?	?	o	+++	o
Mucin. Human placenta	.....	.....	.....	+++	+	.....	+++	Trace
Gelatin. Human placenta	.....	.....	.....	+	+	++	+	+

Mucin was prepared from the cords, which had been washed free from blood, finely ground, and extracted with N/10 sodium carbonate for 24 hrs., strained through cloth, and the residue re-extracted 24 hrs. The total extract was then precipitated with N/10 acetic and filtered, washed, and dried in vacuum over sulfuric acid. After thorough drying all these preparations were kept in tightly stoppered bottles in a dry place. The most important of them are characterized briefly in Table 1.

The unreliability of the conventional methods used in the preparation of "nucleoproteins" is well shown by the very irregular character of the material thus obtained, as indicated in Table 1.

Compare, for example, Preparations B^1 and B^2 of nucleoprotein of cow liver. The first represents the first precipitate obtained from a slightly alkalin extract of cow liver, and the second (B^2) is from the same material on redissolving, filtering, and reprecipitating 5 times. It will be noticed that the proportion of purine has been reduced, and the phosphorus increased, during the "purification." Also note that a carefully purified preparation from dog liver yielded only a trace of purine nitrogen, altho made by the usual routine. The phosphorus is very low in all.

Having obtained our materials (the difficulties of this procedure deserve considerable emphasis), and having studied to some extent their chemical composition and reactions, their biological properties were investigated as follows:

ANAPHYLAXIS.

The materials were tested by this method as it readily gives an indication of antigenic properties and specificity. The results of these experiments, given in Table 2, show definitely that the nucleoprotein fraction of placenta is not a good sensitizer, and that no definite symptoms could be elicited on injecting a second dose of the same material after about 20 days' incubation, altho symptoms of varying degrees of severity were obtained on the injection of human serum or the globulin and albumin fractions of placenta into pigs sensitized with nucleoprotein. Furthermore, pigs sensitized to nucleoprotein and failing to react with the same, reacted to human serum the following day. This is in accord with the work of Pearce, Karsner, and Eisenbrey, and of Wells, to which reference has been made. It is noted that positive results were obtained by this method with the globulin and albumin fractions, but that the intoxications were, if anything, more severe when the second dose was human serum rather than the purified protein. In brief, the manifestations were those of a "general human reaction" with no evidence of specificity of the proteins themselves. The nucleoprotein fraction was inert both in sensitizing and in intoxicating properties, particularly the latter. This is not surprising, in view of the fact that the protein part of the nucleoprotein, providing we have such a body, is probably a histone or a protamine,

TABLE 2.

ANAPHYLAXIS EXPERIMENTS WITH PROTEINS.

	Sensitizing Dose	Amount	Second Injection	Amount	Reaction
	NUCLEOPROTEINS FROM	gm.		gm.	
1.	Sheep liver	0.025	Sheep liver nucleoproteins	0.1	Negative
2.	" "	0.010	" " "	0.1	"
3.	" "	0.005	" " "	0.1	"
4.	Dog liver	0.025	Dog " "	0.1	"
5.	" "	0.010	" " "	0.1	"
6.	" "	0.005	" " "	0.1	"
7.	Human placenta	0.05	Human placental nucleoprotein	0.1	"
8.	" "	0.01	" " "	0.1	"
9.	" "	0.005	" " "	0.1	"
				c.c.	
10.	" "	0.05	Human serum	1	Slight
11.	" "	0.01	" "	1	Moderate
12.	" "	0.005	" "	1	Severe
				gm.	
13.	" "	0.001	Human placental nucleoprotein	0.05	Negative*
14.	" "	"	" " "	"	" *
15.	" "	"	Human placental globulin	"	Slight
16.	" "	"	" " "	"	Doubtful
17.	" "	"	Human placental albumin	"	Slight
18.	" "	"	" " "	"	Moderate
19.	Cow liver B^1	0.005	Cow liver nucleoprotein	0.07	Slight
20.	" "	"	" " "	0.07	Slight
21.	" "	"	" " "	0.05	Negative
22.	" "	"	" " "	0.05	"
				c.c.	
23.	" "	"	Beef serum	1	Slight
24.	" "	"	" "	"	Negative
				gm.	
25.	Cow liver B^2	"	Cow liver nucleoprotein B^1	0.07	Slight
26.	" "	"	" " "	"	Severe
27.	" "	"	" " " B^2	0.05	Negative
28.	" "	"	" " " "	"	"
				c.c.	
29.	" "	"	Beef serum	1	Slight
30.	" "	"	" "	"	"
		c.c.		gm.	
31.	Beef serum	0.01	Cow liver nucleoprotein B^1	0.07	Slight†
32.	" "	"	" " "	"	" †
33.	" "	"	" " " B^2	0.05	Negative†
34.	" "	"	" " "	"	"
35.	Human serum	0.1	Human placental nucleoprotein	0.1	"
36.	" "	0.01	" " "	0.05	"
37.	" "	"	" " globulin	"	Moderate
38.	" "	"	" " albumin	"	Severe
		gm.			
39.	Human placental globulin	0.001	" " nucleoprotein	"	Doubtful
40.	" " "	"	" " "	"	"
41.	" " "	"	" " globulin	"	Severe
42.	" " "	"	" " "	"	"
43.	" " "	"	" " albumin	"	"
44.	" " "	"	" " "	"	"
45.	" " "	"	" " "	"	"
46.	Human placental albumin	"	" " nucleoprotein	"	Slight
47.	" " "	"	" " "	"	Moderate
48.	" " "	"	" " globulin	"	Doubtful
49.	" " "	"	" " "	"	Slight
50.	" " "	"	" " albumin	"	Severe
51.	" " "	"	" " "	"	"
52.	Human mucin from cord	"	" mucin, cord	"	Slight
53.	" " " "	"	" " "	"	Moderate
54.	" " " "	"	Pig stomach mucin	"	Negative‡
55.	" " " "	"	" " "	"	" ‡

* Exper. 13–14 later gave definite reaction to human serum.

† Exper. 31–34 later gave some reaction to beef serum.

‡ Exper. 54–55 next day gave definite reaction to human serum.

both of which are known to be poor antigens or non-antigenic, and the nucleic acid has no antigenic properties.

THE PRECIPITIN COMPLEMENT FIXATION AND PASSIVE ANAPHYLACTIC REACTIONS.

Preparation of the immune sera.—Three pairs of rabbits were injected with nucleoprotein, albumin, and human serum respectively. To the first pair, a total of 2.8 gm. was given in 7 injections intraperitoneally at intervals of 3–4 days. The animals showed no symptoms of toxicity at any time. To the second, a total of 4 gm. was given in 8 doses at the same intervals, by the intravenous method. The first pair treated in this way died on receiving the third dose, with symptoms of acute anaphylactic shock. No evidence of infection could be found on autopsy. Another pair was injected intraperitoneally with no apparent bad results to the animals. The third pair received from 30 to 40 c.c. of human serum in 5 doses by both methods. In all cases the animals were bled to death from the carotid, under ether anesthesia, about 10 days after the last injection; the blood was allowed to clot and the serum was pipetted off and hermetically sealed in tubes. Part was then inactivated, and part kept unheated, all being kept in the refrigerator. The antihuman serum retained its titre almost unimpaired, as shown by both the complement fixation and precipitin tests, for more than a year. The other antisera all kept perfectly also. In the case of one tube of antiserum, however, which contained some red cells it was found that hemolysis was completely or nearly inhibited, which shows the importance of controls for every dilution. The same phenomenon has been noted by Giampalmo.[1] This shows also the importance of having the immune serum perfectly free from red cells, especially when it is allowed to stand so that autolysis may occur.

In the preparation of the hemolytic serum, another interesting point was noted. At the time one of the rabbits was killed she was found to be in advanced pregnancy. After bleeding the mother thoroughly from the carotid, one of the fetuses was carefully removed, its serum obtained and tested with that of the mother.

[1] *Arch. Ital. de Biol.*, 1911, 56, p. 182.

The serum of the fetus gave complete hemolysis in 1:1,000; that of the mother in 1:2,000. The passing over of antibodies through the placenta has long been known, but the amount is seldom stated.

PROPERTIES OF THE VARIOUS ANTISERA.

1. *Precipitin test.*—Precipitins were found in all the antisera prepared, but the antiserum for nucleoprotein was much weaker than the others, that is, only a slight precipitate with a nucleoprotein in a dilution of 1–10,000, and its specific antiserum, while a considerably heavier precipitate was obtained with the same antiserum and human serum diluted 1–40,000, or with the albumin fraction of human placenta. Antiserum to placenta albumin gave quite the same precipitin reactions as the antiserum for nucleoprotein.

Beebe's work[1] with the nucleoproteins on other organs, on the contrary, appears to show specificity. It will be noted, however, that in the protocols given by Beebe the reaction of the immune serum to the serum of the same animal is not shown, nor are the dilutions, etc., given in which the reactions occurred. Pearce[2] was unable to show specificity with any of these fractions by the precipitin reaction.

It is important to run a large number of controls in all this work. Every tube with a different strength of antigen must be run in duplicate against normal rabbit serum. Table 1 shows only the results. Table 3 gives one titration complete as a sample.

Normal serum and antiserum were always used in 0.1 c.c., the several antigens in variable amounts. Readings were taken after 2 hours' incubation and again after standing in the icebox.

2. *Complement fixation.*—Also by this reaction there is no evidence of the specificity of placental nucleoprotein immune serum, for while it reacts with nucleoprotein in 1:100,000 dilution, it also reacts with the globulin fraction and the albumin fraction in just as high dilution, and with human serum in 1:1,000,000 dilution. The antisera for other fractions of placenta proteins also fail to show any specificity, reacting well with high dilutions of human serum;

[1] *Jour. Am. Med. Assn.*, 1910, 55, p. 1714. [2] *Loc. cit.*

and the antiserum for globulin reacts with the albumin as well as with itself, the antialbumin serum and globulin reaction being equally delicate.

These results may be compared with the only other recorded experiments with placental nucleoprotein, namely, those of Frank. He obtained no complement fixation reaction with the serum of rabbits immunized to the nucleoprotein. The antiserum which he obtained by immunizing with suspension of placental cells gave a weak general species reaction, without evidence of specificity for the placenta itself; i.e., much the same result as we obtained with antisera for isolated placenta proteins.

TABLE 3.

PRECIPITIN TEST WITH NUCLEOPROTEIN ANTISERUM.

DILUTION OF ANTIGENS	NUCLEOPROTEIN		GLOBULIN		ALBUMIN		HUMAN SERUM	
	Antiserum	Cont.	Antiserum	Cont.	Antiserum	Cont.	Antiserum	Cont.
1:400.....	Turbid	Clear	Sl. turbid	Clear	Heavy ppt.	Heavy	Ppt.	Clear
1:2,000.....	"	"	" ppt.	"	Med. ppt.	Med. ppt.	"	"
1:4,000.....	Ppt.	"	Def. ppt.	"	Light "	Light "	"	"
1:10,000.....	Sl. ppt.	"	" "	"	Trace	Trace	"	"
1:20,000.....	Trace ?	"	" "	"	"	"	"	"
1:40,000.....	Clear	"	" "	"	Def. ppt.	Clear	"	"
1:100,000.....	"	"	" "	"	Clear	"	Clear	"
1:200,000.....	"	"	Sl. ppt.	"	Sl. ppt.	"	"	"
1:400,000.....	"	"	Clear	"	Clear	"	"	"
No antigen...	"	"	"	"	"	"	"	"

3. *Passive anaphylaxis test.*—Inasmuch as this test was used in checking up the reactions of immune sera prepared for some of the vegetable proteins (see p. 364), with good results, it seemed well to use it here for comparative purposes. The test was carried out as follows: Guinea-pigs of about 300 gm. are given injections of from 1 to 3 c.c.of the immune serum and 24–72 hrs. later an intoxicating dose of the specific antigen, when, in case of a positive result the typical symptoms of acute anaphylaxis will follow. The guinea-pigs receiving serum from a rabbit immunized with nucleoprotein showed no symptoms whatever when given an injection of the same nucleoprotein. The animals sensitized with antialbumin serum did not react to a toxic dose of nucleoprotein, but did react to both the globulin and albumin fractions of placenta. The chief

results obtained with the antisera described above are summarized in Table 4.

Had the results been more favorable up to this point, it was the intention to try the other tests commonly used as well, i.e., agglutination and lysis of cell suspensions, and injections of specific antisera into animals. From the work of all the other observers, however, apparently no data of importance have been gained by the agglutination tests that are not also obtained with the reactions

TABLE 4.

GENERAL REACTIONS OF THE ANTISERA.*

	Antigens	Antisera				
		Antiserum for Egg White	Antiserum for Human Serum	Antiserum for Placental Nucleo-protein	Antiserum for Placental Albumin (A)	Antiserum for Placental Albumin (B)
Precipitin reaction	Egg white	1:200,000				
	Human serum		1:100,000	1:40,000	1:100,000	1:400,000
	Placental nucleoprotein		1:100 (?)	1:10,000	Negative	Negative
	" globulin		1:100,000	1:100,000	1:10,000	1:8,000
	" albumin		1:10,000	1:200,000	1:40,000	1:80,000
	Mucin from cord		1:100			
Complement fixation	Egg white	1:1,000,000				
	Human serum		1:1,000,000	1:1,000,000	1:1,000,000	1:500,000
	Placental nucleoprotein		1:10,000	1:100,000	1:50,000	1:100,000
	" globulin		1:100,000	1:100,000	1:50,000	1:250,000
	" albumin		1:100,000	1:100,000	1:500,000	1:100,000
	Mucin from cord		1:10,000			
Passive Anaphylaxis	Egg white	Severe				
	Placental nucleoprotein			Negative	Negative	
	" globulin				Moderate	
	" albumin				Moderate	

* The figures indicate the highest dilutions, in the case of the precipitin reactions, of the antigens, and in case of complement fixation, of the antisera in which the reactions are positive.

that we have used. Further, it hardly seems that the agglutination tests can be as delicate as the complement fixation tests. In the case of cholera and typhoid, where the agglutination tests are largely used, it is more because of the ease with which they are carried out. As a matter of fact, the other tests would probably be far more delicate. With the placenta it would be hard to get a uniform cell suspension, and besides it would seem that the results are about as definite without this.

In regard to testing the specificity of the antiserum by injecting it into animals and seeking for specific lesions, it would seem rather

crude to attempt this with a serum for which it is impossible to show specificity by such delicate methods as those already used.

CONCLUSIONS.

It is believed, therefore, that the following conclusions are warranted:

1. The nature of nucleoproteins in general is not well known; in fact, they may not exist at all as definite and constant chemical compounds, and it is very doubtful if the "nucleoproteins" isolated from tissue extracts correspond to compounds actually present in the living cells.

2. The "nucleoproteins" studied in these experiments, and in the case of the placenta, the globulin, albumin, and gelatin fractions as well, fail to show organ specificity. The reactions obtained may all be accounted for on the basis of general species reaction. Proteins showing independent specificity could not be isolated from placenta.

3. Antisera prepared with the several protein fractions of the human placenta react as well with human serum as with the isolated placenta proteins themselves; and they showed no definite specificity between themselves.

4. From this we conclude that the possibility that immune serum of therapeutic value in chorion-epithelioma can be prepared specifically for the human placenta is, at least at this time, extremely slight.

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ON THE PRODUCTION OF PRECIPITINS.*

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During the study of the production of antibodies under various conditions, some observations have been made on the development of specific precipitins that may be of interest.

Fornet and Müller[1] and others[2] have shown that when rabbits are injected intraperitoneally with increasing quantities of foreign serum on three successive days there results a fairly copious production of precipitins by about the twelfth day after the last injection. I have obtained the same results. Injected intraperitoneally on three successive days with the serum or blood of various animals (dog, horse, hog, beef, chicken, cat, rat, guinea-pig, fish, or man) in increasing quantities, usually 5, 10, and 15 c.c., one day apart, rabbits commonly produce specific precipitin enough by about the twelfth day after the third injection to cause precipitation in dilutions of the corresponding antigenic serum or blood of 1–1,000 and

* Received for publication February 2, 1914.

[1] *Ztschr. f. biol. Technik u. Methodik*, 1908, 1, p. 201.

[2] Bonhoff and Tsuzuki, *Ztschr. f. Immunitätsf., Orig.*, 1910, 4, p. 180; Gay and Fitzgerald, *Univ. Cal. Publ. in Path.*, 1912, 2, p. 77.

upward to 1–20,000 and higher, and in the case of beef serum as high as 1–200,000 (Table 1).

TABLE 1.

SUMMARY OF RESULTS OF RAPID METHOD OF PRECIPITIN PRODUCTION.

Antigen	Quantity, Mode, and Place of Injection	Day of Test (After Last or Single Injection)	Titer
Human serum	5–10–15 c.c., one day apart. Intrap.	12	12,800
" "	" " " " " Intrav.	"	20,000
" "	" " " " " Intrap.	"	20,000
" "	" " " " " "	14	1,600
" "	" " " " " "	"	1,200
" "	" " " " " "	"	1,000
" "	" " " " " "	"	10,000
" blood	30 c.c. Intrap.	"	2,000
" "	" " "	"	16,000
" "	" " "	"	0*
Horse serum	15 " "	12	30,000
" "	5–10–15 c.c., one day apart. Intrap.	"	50,000
" " *	" " " " " Intrav.	"	20,000
" "	30 c.c. Intrap.	"	6,000
" "	" " "	11†	16,400
Sheep "	5–10–15 c.c., one day apart. Intrap.	13	10,000
" blood	" " " " " "	"	51,000
" "	" " " " " "	"	20,000
" "	" " " " " "	"	8,000
" "	30 c.c. Intrap.	14	16,000
Swine serum	5–10–15 c.c., one day apart. Intrap.	13	20,000
Beef "	5–10 " ‡ " " " "	12	200,000
" "	30 c.c. Intrap.	15	16,000
" "	" " "	"	12,800
Rat "	5–10–15 c.c., one day apart. Intrap.	"	2,400
" "	" " " " " "	10	20,000
Cat "	" " " " " "	14	10,000
Guinea-pig serum	" " " " " "	12	1,280
" "	" " " " " Intrav.	13	8,000
Fish "	" " " " " Intrap.	5§	2,560
Chicken "	" " " " " "	7	1,280
" "	" " " " " "	"	1,280
" blood	" " " " " "	13	60,000

* In this animal repeated tests were made during three weeks after the injection, but in no instance was any precipitate detected.

† The rabbit died on the eleventh day.

‡ Single injections of beef serum in quantities of 4–6 c.c. per kilogram of weight of rabbits gave antiserum of titer varying from 500–10,000 on the fourteenth to fifteenth day. The place of injection, whether intravenous or intraperitoneal, seemed to have no influence on this result.

§ This rabbit died on the sixth day.

The amount of antigen introduced by this method of triple injection was 30 c.c. in most cases. This amount of antigen in one injection, judging from a few experiments (Table 1), results in the production of about as much precipitin as when the antigen is given in three instalments one day apart.

The tests were made in small tubes with 0.5 c.c. of the diluted antigen and 0.1 c.c. of antiserum, care being taken to obtain a precise line of contact between the two fluids. The tubes were left at room temperature and the results recorded at the end of one hour. When tests were made with dilutions of whole blood the corpuscles

were first laked by means of water, and the normal salt content restored by the addition of the required amount of physiological salt solution of double strength, further dilutions being made with salt solution of usual strength. To secure perfect clearness filtration was used whenever necessary.

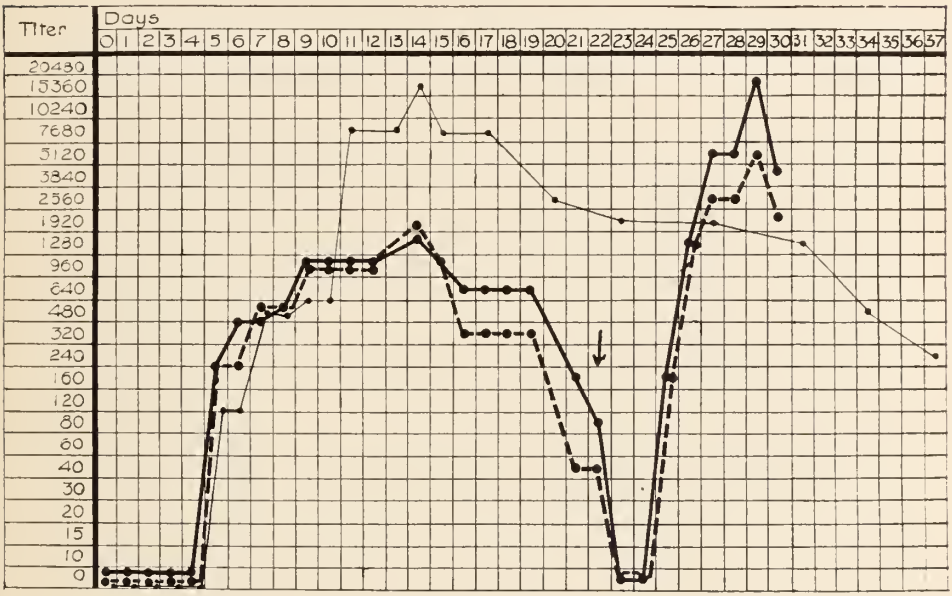

Chart 1.—Curves showing the course of specific precipitin in three rabbits injected with human serum or blood.

The rabbits represented by the heavy lines were injected peritoneally three times—on the first day with 5 c.c., on the second with 10 c.c., and on the third with 15 c.c. of human serum—and reinjected intravenously with 10 c.c. of human serum on the twenty-second day after the third injection. Day 1 is the first day after the third injection.

The figures under "titer" give the highest dilution of human serum in which the rabbit serum produced precipitate.

The rabbit represented by the fine line received 30 c.c. of defibrinated human blood on 0 day. In this case the figures under "titer" give the highest dilution human blood in which the rabbit serum produced precipitate.

It is obvious that under certain circumstances the rapid method of injecting antigen may have advantages of a practical nature over the older method of introducing the antigen at longer intervals. When old and young, healthy and more or less diseased, animals are used indiscriminately, both methods are subject to individual variations in the power to produce precipitins, but the rapid method

seemingly to no greater degree than the other. At all events, the fact that certain rabbits are found to produce comparatively little precipitin, no matter what method is used, makes it advisable always to immunize several at the same time, especially in case antihuman precipitin is to be produced as the response to human antigen occasionally may be comparatively slight.

The injection of the whole blood appears to produce just as much if not more precipitin than the injection of serum alone,

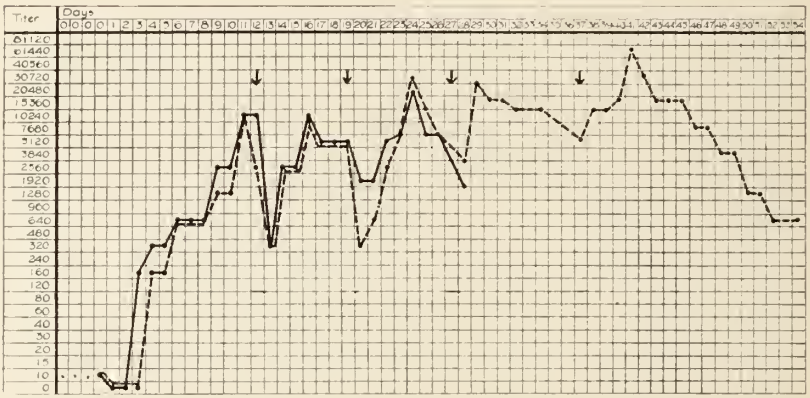

CHART 2.—Curves showing the course of specific precipitin in two rabbits injected with human serum and blood.

Three injections were made intraperitoneally of 5, 10, and 15 c.c. of human serum on three successive days respectively.

Day 1 on the chart is the first day after the last injection. On the twelfth day each received 20 c.c. of defibrinated human blood intraperitoneally; on the nineteenth, 30 c.c.; on the twenty-seventh, 40 c.c., and two days later one rabbit died; on the thirty-seventh day the remaining rabbit received 50 c.c. of human blood intraperitoneally.

The figures under "titer" represent the highest dilution of human blood in which precipitate was produced by the immune serum.

especially when tests are made with dilutions of whole blood. As we deal usually with whole blood rather than with serum only, in the identification of blood spots, it seems that it usually would be of advantage to use an antiserum produced by injection of the whole blood.

That the injection of carefully washed human corpuscles, which is made in order to obtain hemolytic amboceptor, also results in the production of precipitin for proteins in human serum (and blood) is shown by the following observations:

Case 49.—This rabbit received intraperitoneal injections of carefully washed human corpuscles as follows: April 1, the corpuscles of 3 c.c. of blood; April 6, of 5 c.c.; April 11, of 7 c.c.;

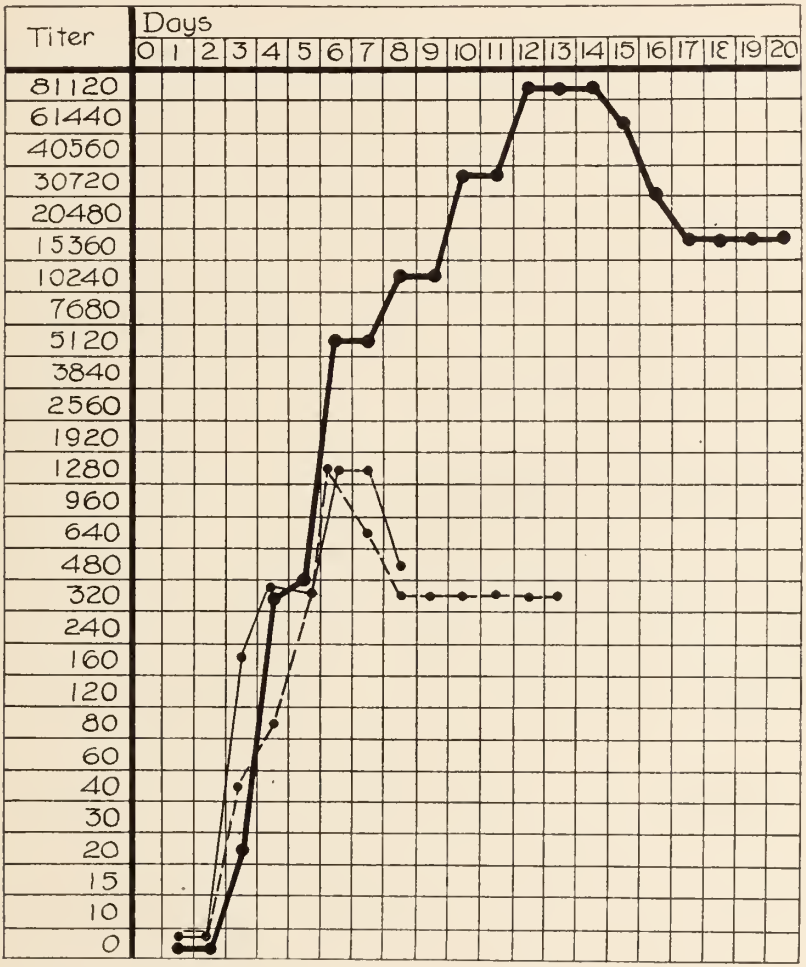

Chart 3.—Curves showing course of specific precipitin in rabbits injected with chicken blood or chicken serum

The fine lines refer to two rabbits, each of which was injected intraperitoneally with 5, 10, and 15 c.c. of chicken serum one day apart. The heavy line refers to a rabbit injected in the same way with the same quantities of defibrinated chicken blood.

Day 1 is the first day after the last injection.

The figures under "titer" give the highest dilution of chicken blood in which the immune serum produced precipitate.

April 16, of 9 c.c., and April 21, of 11 c.c. The titer of the serum on April 29 was 128,000 (human serum).

Case 61.—This rabbit received intraperitoneally the washed corpuscles of 5, 10, 15, and 20 c.c. of human blood in four injections

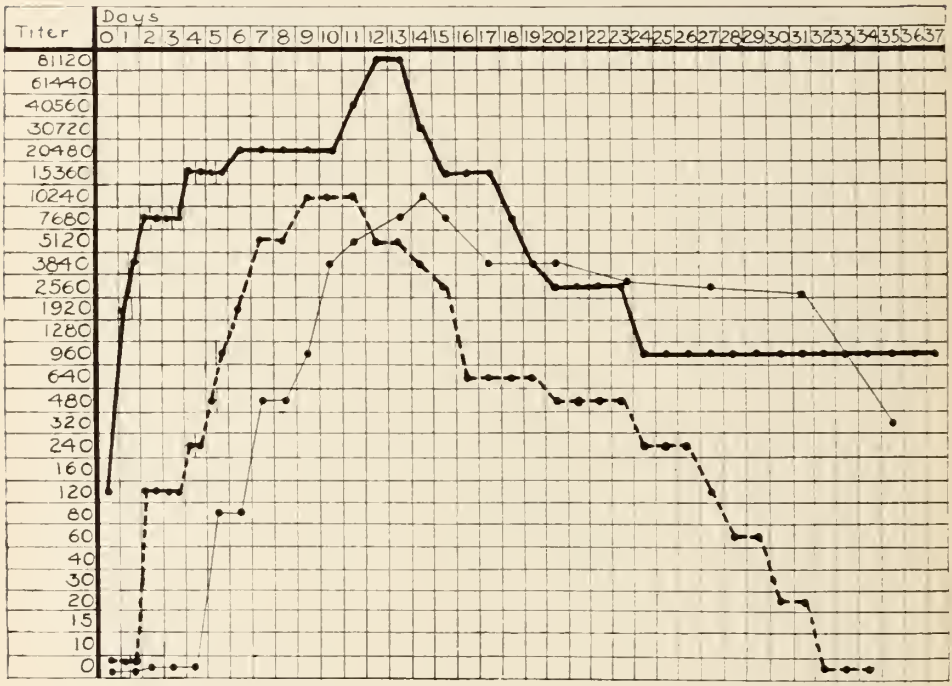

CHART 4.—Curves of precipitin and lysin and of precipitin in two rabbits injected with sheep blood.

Heavy solid line = lysin, heavy broken line = precipitin in rabbit injected intraperitoneally with 5, 10, and 15 c.c. sheep blood, one day apart. On the first five days two daily determinations were made about twelve hours apart. Day 1 is first day after last injection.

Fine line = precipitin in rabbit injected intraperitoneally at one time (day 0) with 30 c.c. of sheep blood.

The figures under "titer" give in the case of precipitin the highest dilution of sheep blood in which precipitate was produced by the rabbit serum; in the case of the lysin, the highest dilution in which the rabbit serum produced distinct lysis of sheep corpuscles.

In testing for the lysin, the rabbit serum was first heated at 58° C. for 30 minutes: the total quantity of each mixture was 0.6 c.c. of which 0.2 c.c. consisted of a 5 per cent suspension of washed sheep blood and 0.007 c.c. of fresh guinea-pig serum (complement), the rest being rabbit serum and salt solution.

four days apart. When tested with human serum ten days after the last injection the titer of the antiserum was 100,000.

When the course of the development of specific precipitin in the blood of rabbits immunized by the rapid method is followed by

daily tests under as strictly comparable conditions as possible, a curve is obtained that in all essentials corresponds to the typical curve described by the antibodies that develop after the single injection of other antigens under suitable conditions.

As shown by the charts, the acme is reached about the twelfth day or thereabouts after the last injection in the case of the triple

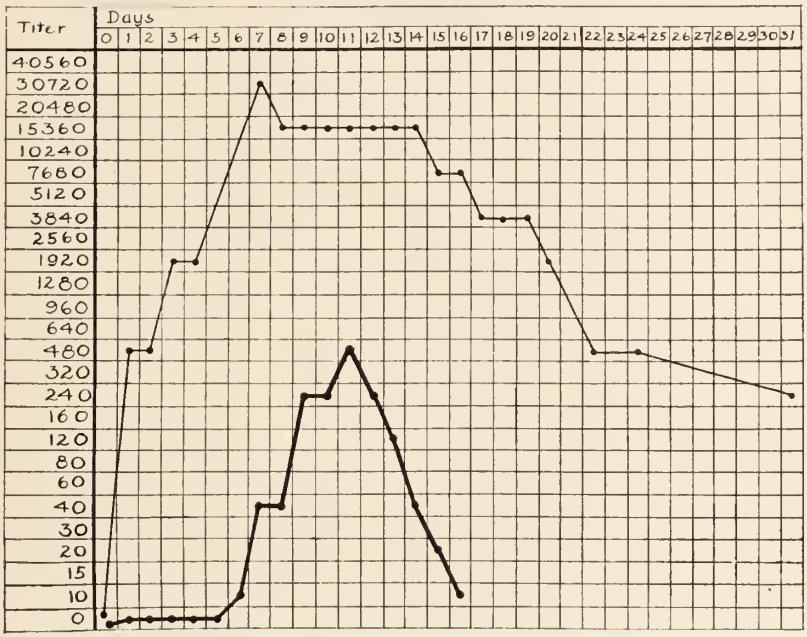

Chart 5.—Curves of precipitin (heavy line) and lysin (fine line) in dog injected with goat blood. Three injections were made intraperitoneally—on the first day, 15 c.c., on the second, 30, and on the third, 45.

The figures under "titer" give in the case of precipitin the highest dilution of goat blood in which precipitate was produced by the dog serum; in the case of the lysin the highest dilution in which the dog serum produced distinct lysis of goat corpuscles.

In testing for the lysin, the dog serum was first heated at 58° C. for 30 minutes; the total quantity of each mixture was 0.6 c.c., of which 0.2 c.c. consisted of a 5 per cent suspension of washed goat blood and 0.0125 c.c. of fresh guinea-pig serum (complement), the rest being dog serum and salt solution.

method of injection, and as a rule a day or two later in the case of the single injection. The earliest apparent increase occurs rather abruptly, that is, in the course of a few hours, and most frequently on the third or fourth day, but sometimes a day or so earlier or later. There now comes a rapid rise at an increasing rate day by day until the acme is attained, whereupon there follows a gradual

decline. As shown in Chart 4, there may be a considerable rise in the course of 12 hours. When the curve remains low the period of latency may be prolonged.

Occasionally the curve without any apparent cause (infection?) begins to recede much earlier than is usually the case (see Chart 3).

As illustrated in Charts 1 and 2, subsequent injections of antigen may cause prompt decline in the amount of free precipitin in the blood, which is followed by an increase, the high point of which is often higher than the previous high point.

Chart 4 illustrates the fact that by means of the methods used the lysin is demonstrable earlier than the precipitin when the rabbits are injected with sheep blood, and also persists longer. Otherwise the courses of the two curves appear to be parallel.

The dog is known to be a poor producer of precipitin as compared with the rabbit, and this fact is illustrated in Chart 5, which gives the determination of the specific precipitin and lysin in a dog injected with goat blood.

SUMMARY.

By giving rabbits intraperitoneal injections of increasing quantities of serum or blood on three successive days a serviceable precipitating serum may be produced in about 15 days. The same quantity of antigen injected at one time also appears to give good results. The curve of the precipitin in such cases is like the simple antibody curve following a single injection of other antigens.

The injection of whole blood may be more advantageous in producing more precipitins for blood proteins in general than the injection of serum only.

The injection of washed human corpuscles gives rise to precipitins for human serum.

THE COLON GROUP OF BACTERIA.*†‡

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INTRODUCTION.

The presence of gas-forming bacteria in milk is usually considered an indication of fecal contamination. This is based on the assumption that all, or nearly all, of the gas-forming bacteria likely to be detected in milk by the ordinary methods of study are of the group having their habitat in the digestive tract of warm-blooded animals and finding little opportunity for multiplication under other conditions. In many board of health laboratories the determination of gas formers of the colon type is one of the routine examinations of milk, and the milk is judged largely by the results of this test.

When milk is used for cheese making the gas-forming bacteria become of economic importance because the gassy fermentation may be carried to the cheese and the product damaged by the bad flavors accompanying gassy fermentations as well as by the appearance of the cheese.

Notwithstanding the importance of the group, our knowledge of the gas-producing bacteria is very fragmentary and confusing. This knowledge has served a very useful purpose, but the time has come when, to make real progress, it is essential that we have some exact information on the various groups of bacteria concerned in the fermentations of milk, their origin, the exact nature of the changes they produce, and their relation to one another. We need

* Received for publication October 28, 1913.

† Published by permission of the Secretary of Agriculture.

‡ Some months after the preparation of the manuscript of this paper, appeared Frieber's article. "Die Bedeutung der Gasabsorption in der Bakteriologie," *Centralbl. f. Bakteriol.*, Abts I, *Orig.*, 1913, 69, p. 437 We are pleased to note the substantial agreement between our judgment and that of Frieber in regard to the untrustworthy nature of almost all the bacteriological gas analyses recorded in the literature. Some of the literature which we have mentioned has been covered in greater detail by Frieber, but our manner of dealing with it will be found to be a valuable supplement to Frieber's treatment.

In regard to Frieber's improvement upon the Hofstädter apparatus we must conclude, both from a theoretical consideration and from a study of the experimental results, that it still possesses defects which militate against its value for exact work.

more exact knowledge of the physiological process by which bacteria change the appearance and composition of the medium in which they live. This can come only through laborious and extensive investigations covering a large number of typical cultures. To this end many of the slipshod methods which have found their way into bacteriological technic must be displaced by the exact methods of the chemist. This is especially evident in the biochemical tests, the reduction of nitrates, the digestion of proteins, the formation of indol, and the fermentation of carbohydrates and other test substances which have been generally used in the differentiation of bacterial species.

In but few of the tests has exact analysis an honorable place. This is unfortunate. So long as an inaccurate method is employed the observer is inevitably induced to become careless, and he is tempted to rely upon the result of a statistical treatment of many determinations, forgetting the proper relationship between mathematics and data expressed in John Hopkinson's famous sentence: "We must remember that we cannot get more out of the mathematical mill than we put into it, altho we may get it in a form infinitely more useful for our purpose." But perhaps the most unfortunate consequence of the collection of inaccurate data is that the wide discrepancies destroy that faith in the definiteness of physiological processes which is the inspiration of those who hope to see bacteriology become a more exact science.

Qualitative differences, such as the fermentation of lactose by one organism and the failure of another, are doubtless distinct and positive in most cases. And yet the end products formed in the fermentation of carbohydrates by certain large classes of bacteria display a significant monotony, which should make one pause to consider whether some of the reactions involved are not common to all, whether some qualitative distinctions are not false, and whether any system of classification which pretends to differentiate individual cultures of a group can have a logical foundation or a permanent position unless it is founded upon quantitative data.

Notwithstanding the great accumulation of descriptions of bacterial species and varieties, it is difficult and in many cases impossible to identify any but the few which have been extensively

studied on account of their great importance as disease producers. In many cases, even these have varieties, subgroups, and atypical strains separated from the type by characters of doubtful significance. This is especially true of the colon group, a large family with very indefinite limits, radiating in all directions from the type organism, the *Bacillus coli communis*. Notwithstanding the fact that this organism is practically a normal inhabitant of the digestive tract and is found frequently in water, milk, and other material likely to become contaminated with feces, it is very difficult to establish the line of demarkation which separates *B. coli communis* from *B. lactis aerogenes* or others of its near relatives.

However imperfect may be our present methods of bacterial classification, there can be no question of the desirability of an accurate knowledge of the various important groups of bacteria whose existence is now recognized, in order that the limits of each group may be ascertained, the naturally related species established, their normal habitat worked out, and their various physiological functions studied in detail. Unfortunately, much of the description and naming of species has preceded the accurate study of the larger groups and the knowledge of the characters which for this particular group are significant and of value in determining the natural subdivisions. Bacteriologists have been much in the position of a biologist who casts a line in new waters, catches one fish, and uses this single specimen as the basis of a description of a new species. He may by chance have the type of a new species, or it may be an individual dwarfed by unfavorable conditions or changed by an unusual habitat. The bacteriologist who fishes a single culture from the unknown waters of the microscopic world has less chance of securing a type specimen, because he is working with organisms whose simple structure allows them to adapt themselves readily to new conditions and the number of varieties is correspondingly great.

The group in question, that usually designated as the colon group, is an excellent example, especially in its relation to water and milk, of the great need of exact work on an entire group. We have various names used to denote different members of the group, but the characters of each are by no means definite, and there is lit tle

evidence that the differentiation follows natural lines; we have tests drawn so closely that, presumably, they differentiate between strains of recent and remote fecal origin, but the tests are based on reactions of admittedly doubtful stability.

A study of the gas-forming bacteria of milk is essentially a study of this group. We have attempted to determine in some measure the significant characters which may be used in determining the natural limits of the group and the subdivisions which have arisen by descent from a common ancestor. We have given especial attention to the gas-producing function and its variations when determined by exact methods. This has been supplemented by determinations of the ability to ferment various carbohydrates, alcohols, and glucosides, the liquefaction of gelatin, the production of indol, and the reduction of neutral red and of nitrates. Finally, the results of these observations have been brought together and their relations to each other studied in an attempt to establish some, at least, of the natural families which it is believed must exist in a group of this size.

RESULTS OBTAINED.

Cultures studied.—The cultures used in this work were practically all obtained from milk or milk products, such as cheese and ice cream. Geographically they represented a wide distribution, including cultures obtained from Albert Lea, Minnesota; Madison, Wisconsin; Chicago, Ithaca, Boston, New York City, Baltimore, and Washington. The collection also contained a few cultures of established identity which were obtained for comparison. These included:

B. coli communis (*em*), a very old culture obtained from Professor C.-E. A. Winslow's collection in the American Museum of Natural History and coming originally through Dr. D. D. Jackson from the Kral collection.

B. aerogenes (*el*) from the same source.

B. coli communis (*fg*) from the Hygienic Laboratory of the U.S. Public Health Service.

B. coli communis (*fj*) from the laboratory of the University of Wisconsin Agricultural Experiment Station.

Bacillus of dysentery (Bitular) (*ev*) from the Biochemic Division of this bureau.

B. cholera suis (*ew*) isolated from a guinea-pig in the Biochemic Division of this bureau.

B. phytophytorus (*fp*), a very old culture coming originally from Apple and obtained from the laboratory of Plant Pathology of the Bureau of Plant Industry.

The usual procedure in isolating gas-forming cultures from milk was to plate the milk on lactose agar, incubate at 30°, and inoculate a large number of the resulting colonies in dextrose broth tubes with inverted inner tubes. All of these that showed

gas were replated and agar slope cultures made from the colonies. Usually only 2 or 3 cultures were made from one sample.

CHARACTERS STUDIED.

The cell.—These cultures showed so little variation in morphology that no attempt was made to use this character in differentiation. With possibly a few exceptions they all agreed with the short thick rod typical of the colon group. Spore formation was determined by staining and by exposing the culture to a temperature sufficiently high to destroy non-spore-forming cells. In nearly all cases these results were negative, but with a few cultures the results were uncertain. Nearly all of the cultures were gram negative, but as is well known this reaction is subject to some variation, and not infrequently the preparation showed both positive and negative cells.

Motility was not determined as it was believed that this character is too subject to variation owing to slight changes in the conditions. Burri and the earlier workers on this group placed much dependence on motility but in recent years it has been generally disregarded. Burk found it very difficult to determine motility with any certainty. Howe concluded that motility is not significant in the colon group. Jaffe says that motility is of no value in separating varieties of the group. Savage thinks that all true coli are motile but that when freshly isolated the motility may fail. Motility when observed may be additional evidence to assist in placing the culture but its failure is merely negative evidence.

Indol production.—While the production of indol is usually considered as one of the distinguishing characters of the colon group, it is recognized that it is subject to variation, and a negative test should not necessarily prevent a culture from being classed as *B. coli*. The Committee on Standard Methods of Water Analysis of the American Public Health Association makes the statement that indol production and nitrate reduction are variable and in a later report indol production is not included in the characters of the colon group altho it is used in the differentiation of some of the varieties. The indol test was made in the usual way on cultures incubated 7 days at 30° C. The results are given in Table 2.

Reduction of nitrates.—What has been said of the formation of indol is true also of the reduction of nitrates. The test is considered inconstant and of doubtful diagnostic significance.

This test was applied to cultures grown at 30° C. for 7 days in the following medium: peptone, 1.0 gm.; potassium nitrate, 0.2 gm.; water distilled, 1,000 c.c. The results are tabulated in Table 2.

Reduction of neutral red.—This test was found to be of some value in the differentiation of the lactic acid bacteria. Its value for other groups is doubtful, but all cultures were tested and the results are given in Table 2. To 1,000 c.c. of neutral broth were added 5 gm. of dextrose and 10 c.c. of 0.5 per cent solution of Grübler's neutral red. The neutral broth was made as follows: beef extract, 4 gm; peptone, 10 gm. and water, 1,000 c.c.

The tubes were examined after 7 days' incubation at 30° C. in an anaerobic jar from which the oxygen was absorbed with pyrogalic acid.

Liquefaction of gelatin.—The growth on gelatin and the liquefaction of the medium has always been considered of the greatest importance in differentiating bacteria, but in recent years there has been a growing tendency to attach less significance to the character of the growth or nature of the liquefaction and to depend on the fact of

liquefaction or non-liquefaction for identification, notwithstanding the generally recognized fact that this character is subject to considerable variation. In our own cultures it has been observed that several cultures giving a distinct liquefaction on the first test failed entirely on the second, a few weeks or months later. Of course in these cases the possibility of contamination was not absolutely excluded but it is not unlikely that the change was an instance of loss of function. The liquefaction of the gelatin is the expression of the attempt by the cell to secure nitrogenous food by the excretion of a proteolytic enzyme. When soluble nitrogen is supplied in excess of the needs of the organism the enzyme is no longer useful and it is not surprising that it is lost after a few generations on artificial media. In the fermentation of sugars, on the other hand, we have a function more closely associated with the protoplasm of the cell and probably with the production of energy essential to the activity of the cell and therefore less likely to variation.

We have used the method first suggested by Clark and Gage, and used by the Winslows in their work on the coccaceae and by us in the work on the lactic acid bacteria. This consists in inoculating the surface of a gelatin tube with a few drops of fluid culture, marking the top of the gelatin on a strip of paper pasted on opposite sides of the tube, and measuring the liquefaction after 30 days' incubation at 20° C. The results are given in Table 1.

TABLE 1.

Liquefaction of Gelatin.

Total Cultures	0 mm.	0–5 mm.	6–10 mm.	11–15 mm.	16–20 mm.	21–25 mm.	26–30 mm.	31–35 mm.	36–40 mm.	41–45 mm.	46–50 mm.	Over 50 mm.
122	109	1	0	0	1	4	3	0	1	0	1	2

These results will be discussed under another head.

Fermentation of carbohydrates.—The distinguishing characteristic of the gas-forming bacteria is their ability to form gas, acids, and other by-products from various carbohydrates, alcohols, and glucosides, and it is probable that in these reactions is found the most substantial basis for subdivisions of the group. The value of any reaction for this purpose depends (1) on its usefulness in showing lines of natural relationships, and (2) on its stability. It may be assumed that a character showing natural relationship would be stable since stability comes through repetition in many generations. There is, or has been, considerable difference of opinion in regard to the stability of the various manifestations of fermentative ability in bacteria. Not a few writers have asserted that the physiological reactions in general and the fermentation of sugars in particular are too variable to be used for purposes of classification. Burri found that old colonies frequently developed cells capable of producing gas from sugars not fermented by younger colonies. Gas formation was determined by shake agar cultures. Revis believes that physiological properties may be lost or acquired under action of competition and that a variation may become suddenly fixed. He found that when a typical *B. coli* was grown in a broth containing malachite green it gradually lost the power of forming gas altho it grew luxuriantly and typically on solid media. This new variety seemed to be permanent. Penfold in a series of papers shows the possibility of bacterial mutations produced partly under the influence of chemicals added to the media and partly under normal conditions through the

development of papillae on agar colonies. These new varieties which usually were non-fermenters were said to be permanent. On the other hand, Abbott, who was able to produce variations in *Sta. pyogenes aureus* by exposing repeated generations to various chemicals, found that this variation was in intensity of reactions rather than in the gain or loss of a function and that the sugar-splitting ability was not changed. Bergey using similar methods with *B. coli* was unable to obtain any mutations altho there was some evidence of alteration in some of the immunity reactions. Berry and Banzhof attempted to obtain by selection races of diphtheria bacilli with divergent powers of acid production and found that the strains instead of diverging tended to approach each other. Similar results were obtained by Buchanan and Truax working with streptococci. Revis, who produced atypical varieties by exposing cultures to malachite green, found that in cultures of *B. coli* held several months in sterile soil and in synthetic media there was no loss of any physiological function altho there was some variation in the intensity of the reaction. MacConkey held *B. coli* in water 358 days without change in its characters. MacConkey also gives the results of physiological tests on 15 cultures of *B. typhosus* from different sources including one that had been 16 years on artificial media. All of these cultures gave identical reactions. Similar results were obtained by Harding working with *Ps. campestris.*

The value of much of the work bearing on variations in bacteria, especially variations in the gas-forming function, is diminished by the inexact methods of measuring [illegible] which, as will be shown in this paper, may lead to erroneous conclusions. It should also be remembered that, from the standpoint of systematic bacteriology, the important consideration is not the variations which may be formed by artificial conditions but the variations that occur in nature. If the cultural characteristics of a type organism are found originally by inexact methods, may we with reason say that slight variation from these characteristics is a true indication of variation in the physiological power of the organism? Conversely, if closer scrutiny reveals frequent variation among the cultural characteristics of the same organism, what hope is there of ever establishing tests sufficiently constant to be of diagnostic value?

In this vicious circle the larger question of natural selection and mutation are lost. It would therefore be in a certain sense a test both of the constancy of a particular medium and of a particular culture if it could be demonstrated that with the medium and culture in question the same products were reproducible *quantitatively.*

In dealing with certain questions, quantitative data alone will advance the science of bacteriology just as quantitative data alone have furnished the solution of problems in other sciences after qualitative experiments had plunged the subject into confusion.

Acid formation.—In studies of the bacteria of the colon group acid formation has usually been subordinated to gas formation as a measure of the fermentation of sugars and other test substances. Winslow in his work on the coccaceae has shown the value of the amount of acid formed not only because it gives exact results but also because in many cases varieties may be separated by the relative amounts of acid produced under given conditions. Some bacteria may produce sufficient acid in the absence of a fermentable sugar to affect the reaction of the broth sufficiently to change the color of an indicator and thus give misleading results. This condition is avoided by titration, which allows a distinction between the slight acidity that may come from any one of a variety of by-products and the marked acidity that usually comes from the fermentation of a carbohydrate.

TABLE 2.

General Characteristics of All Cultures.

Culture	Morphology	Spores	Gram Stain	Mm. Gelatin Liquefied in 30 Days at 20°	Indol	Nitrates Reduced	Neutral Red Reduced	Percentage Lactic Acid From												
								Dextrose	Levulose	Galactose	Adonite	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Salicin	Duplicate
a	sr			0				.216	.000	.193	.000	.000	.324	.234	.000	.000	.270	.198	.000	.000
b	sr			24				.265	.207	.310	.000	.400	.198	.468	.090	.000	.234	.310	.468	.000
c	sr			28				.356	.144	.270	.000	.463	.207	.373	.108	.000	.333	.252	.441	.000
f	sr			23				.428	.477	.337	.000	.490	.333	.432	.000	.000	.378	.283	.495	.000
h	sr			23				.342	.468	.351	.000	.411	.072	.432	.126	.000	.360	.364	.459	.000
m	sr			0				.252	.288	.234	.621	.360	.324	.346	.162	.000	.369	.369	.531	.000
n	sr			0				.090	.342	.342	.423	.360	.270	.342	.486	.000	.216	.360	.531	.288
o	sr			0				.275	.378	.319	.540	.400	.373	.437	.477	.000	.306	.117	.540	.369
p	sr			0				.468	.513	.337	.000	.000	.342	.000	.000	.000	.414	.260	.504	.000
s	sr			0				.297	.468	.355	.423	.387	.310	.237	.468	.054	.342	.265	.531	.000
u	sr			0				.360	.540	.180	.297	.468	.153	.318	.342	.000	.333	.265	.567	.000
w	sr			0				.120	.144	.153	.000	.252	.207	.387	.000	.000	.306	.144	.468	.000
ag	sr			0				.108	.198	.126	.423	.306	.279	.252	.133	.000	.288	.117	.369	.000
aj	sr			0				.153	.450	.168	.153	.207	.270	.216	.333	.000	.288	.135	.477	.000
ak	sr			0				.576	.558	.261	0	.387	.333	.504	.000	.000	.405	.117	.369	.450
al	sr			0				.540	.531	.	0	.306	.243	.531	.000	.000	.306	.135	.495	.522
ao	c			0				.000	.153	.	0	.369	.207	.117	.000	.000	.252	.036	.540	.000
ap	sr			0				.513	.108	.	4	.333	.189	.495	.324	.000	.126	.072	.540	.126
aq	sr			0				.495	.540	.	3	.000	.351	.000	.000	.000	.369	.261	.297	.540
ar	sr			0				.486	.531	.	.	.000	.189	.000	.000	.045	.018	.000	.063	.009
aw	sr			0				.576	.441	.	.	.000	.378	.000	.000	.000	.387	.288	.036	.000
ax	sr			0				.576	.549	.	.	.000	.378	.000	.000	.000	.306	.297	.378	.549
ay	c			0				.495	.414	.	.	.000	.324	.000	.000	.000	.369	.126	.486	.423
az	sr			0				.540	.585	.	.3	.000	.360	.000	.000	.000	.495	.144	.369	.000
ba	sr			0				.558	.549	.	.3	.000	.360	.000	.000	.000	.369	.297	.351	.567
bb	sr			0				.540	.531	.	4	.000	.216	.000	.000	.000	.306	.234	.324	.513
bc	sr			0				.621	.549	.	.2	.000	.297	.243	.000	.000	.423	.108	.540	.000
bd	sr			0				.549	.423	.	.3	.000	.351	.000	.000	.000	.432	.252	.000	.000
be	sr			0				.558	.360	.	.	.000	.369	.225	.000	.000	.414	.234	.459	.000
bf	lr			0				.531	.549	.	.	.000	.369	.000	.000	.000	.306	.306	.387	.387
bg	sr			0				.576	.549	.	.	.000	.387	.000	.000	.000	.396	.171	.558	.495
bh	sr			0				.621	.576	.	.	.000	.315	.000	.000	.000	.459	.099	.332	.340
bi	sr			0				.540	.495	.	.	.000	.378	.000	.000	.000	.405	.288	.000	.000
bj	sr			55				.135	.144	.	0	.351	.036	.063	.513	.000	.216	.234	.558	.000

TABLE 2—*Continued.*

Culture	Morphology	Spores	Gram Stain	Mm. Gelatin Liquefied in 30 Days at 20°	Indol	Nitrates Reduced	Neutral Red Reduced	Percentage Lactic Acid From												
								Dextrose	Levulose	Galactose	Adonite	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Salicin	Dulcite
dk	lr			0	+	+	+	.567	.549	.306	.000	.342	.360	.057	.000	.000	.423	.126	.297	.378
dl	r			0	+	+	+	.504	.513	.297	.000	.306	.351	.666	.000	.000	.387	.270	.477	.540
dm	sr			0	+	+	+	.117	.342	.225	.000	.306	.333	.216	.288	.000	.216	.090	.423	.135
dn	sr			0	+	+	+	.081	.168	.117	.000	.288	.306	.207	.045	.000	.315	.207	.549	.000
do	sr			0	+	+	+	.562	.540	.408	.000	.522	.387	.588	.000	.000	.396	.144	.468	.513
dp	sr			0	+	+	+	.414	.090	.102	.405	.378	.261	.234	.351	.000	.387	.045	.531	.090
dq	sr			0	+	+	#	.603	.171	.396	.000	.000	.300	.000	.000	.000	.423	.342	.045	.000
dr	r			0	+	+	#	.549	.522	.297	.009	.000	.360	.000	.000	.000	.423	.261	.387	.000
ds	sr			0	+	+	+	.684	.810	.253		.594	.522	.630	.072	.000	.432	.135	.630	.585
dt	sr			0	+	+	+	.558	.018	.378	.000	.468	.378	.000	.000	.000	.423	.360	.360	.000
du	r			0	+	+	+	.531	.594	.360	.000	.000	.378	.423	.000	.000	.441	.378	.414	.522
dw	r			0	+	+	+	.513	.540	.333	.000	.450	.432	.000	.000	.000	.450	.270	.423	.000
dv	sr			0	+	+	+	.223	.144	.144	.423	.414	.288	.333	.000	.000	.216	.085	.486	.000
dz	sr			0	+	+	+	.000	.225	.102	.000	.153	.288	.113	.000	.000	.270	.108	.558	.000
ea	sr			0	+	+	+	.567	.540	.360	.000	.000	.306	.123	.000	.000	.414	.108	.315	.450
ec	sr			0	+	+	+	.567	.540	.351	.405	.657	.207	.000	.000	.000	.405	.207	.468	.000
ed	sr			0	+	+	+	.558	.531	.324	.405	.000	.324	.000	.000	.000	.351	.117	.459	.072
ee	sr			0	+	+	+	.551	.567	.351	.000	.000	.360	.000	.000	.000	.360	.360	.477	.567
ef	sr			0	+	+	+	.531	.513	.342	.000	.477	.387	.508	.000	.000	.396	.207	.495	.342
eh	sr			0	+	+	+	.504	.387	.360	.000	.000	.270	.144	.000	.000	.387	.252	.009	.513
ej	sr			0	+	+	+	.504	.378	.306	.000	.306	.108	.513	.027	.000	.495	.117	.306	.414
ek	sr			0	+	+	+	.540	.576	.333	.018	.351	.270	.000	.000	.000	.378	.288	.081	.000
el	r			0	+	+	+	.504	.459	.225	.000	.201	.201	.540	.000	.000	.504	.162	.477	.558
em	sr			0	+	+	+	.441	.441	.324	.000	.000	.054	.000	.000	.000	.000	.000	.072	.000
en	sr			5	+	+	+	.541	.000	.324	.000	.351	.144	.207	.000	.540	.225	.009	.504	.207
eq	sr			0	+	+	+	.585	.531	.144	.432	.000	.315	.000	.000	.036	.063	.144	.459	.000
er	sr			0	+	+	+	.594	.558	.207	.459	.000	.342	.117	.000	.000	.567	.072	.018	.000
es	sr			0	+	+	+	.567	.549	.477	.000	.000	.333	.000	.000	.000	.531	.171	.351	.225
et	r			0	+	+	+	.126	.108	.261	.540	.270	.207	.333	.009	.000	.495	.153	.432	.225
eu	sr			0	+	+	+	.531	.531	.477	.000	.000	.342	.000	.000	.000	.522	.135	.360	.000
ev	sr				+	+	+	.126	.126	.162	.297	.168	.252	.315	.000	.000	.378	.102	.306	.000
ew	sr			0	+	+	+	.270	.441	.441	.000	.000	.000	.000	.000	.000	.657	.153	.414	.594
ey	r			0	+	+	+	.000	.045	.180	.000	.153	.234	.126	.027	.000	.162	.144	.486	.000
fa	sr			0	+	+	+	.360	.000	.000		.000	.261	.000	.117	.432	.477	.171	.054	
fb	sr			50	+	+	+	.522	.225	.414	.000		.261	.342	.000	.000	.549	.090	.450	.000
fc	sr			0	+	+	+	.126	.171	.117	.171	.261	.315	.243	.117	.000	.201	.126	.360	.000
fd	sr			0	+	+	+	.225	.252	.225	.000	.117	.279	.306	.018	.000	.324	.108	.324	.000
fe	sr			0	+	+	+	.180	.171	.261	.000	.216	.162	.306	.027	.000	.153	.135	.387	.000
ff	sr			0	+	+	+	.135	.243	.054	.000	.072	.270	.423	.036	.000	.297	.108	.387	.000
fg	sr			0	+	+	+	.225	.468	.450	.000	.144	.253	.324	.027	.000	.396	.270	.333	.324

We have used in our tests for fermentative ability the sugars, dextrose, levulose, galactose, adonite, saccharose, lactose, and raffinose, the polysaccharides starch, and inulin; the alcohols mannite, glycerin, the glucosid salicin and dulcite. These were used in a broth made as follows: beef extract, 0.4 per cent; peptone, 1 per cent; dibasic potassium phosphate, 0.5 per cent, and test substance, 1 per cent. The broth was brought to the neutral point before the addition of the potassium phosphate. The cultures were incubated at 30° C. for 7 days. Some objections may be made to the use of a temperature lower than that ordinarily used with the colon group and one which is below the optimum temperature of many of the cultures. This temperature was selected rather than the more usual one of 37° C., because while many of our cultures grew very slowly at the higher temperature, all grew readily at 30° C. Moreover, the difference in growth at these temperatures is only in rapidity and the maximum acidity, which is reached at either temperature in considerably less than 7 days, is approximately the same in both cases. Five cubic centimeters of the broth were titrated against twentieth normal sodium hydrate after the tubes had been held 15 or 20 min. in a steam bath to drive off as much as possible of the carbon dioxid. The results expressed as percentage of lactic acid are given in Table 2. In all cases the titer of a blank was subtracted from that of the culture.

It was observed that in many cases in which there was a comparatively high acidity in lactose and other sugars which are presumably fermented with some difficulty there was a low acidity and even a neutral or alkalin reaction in the broth containing dextrose, levulose, or galactose. This is due, not to the failure to ferment the sugar, but to the production by some varieties, after the sugar fermentation has ceased, of some substance with an alkalin reaction which in time may entirely overcome the earlier acidity of the culture. This phenomenon is frequently observed in lactose litmus plate cultures of colon organisms. This alkali formation evidently begins after the completion of the acid fermentation and is therefore more evident in a broth with an easily fermentable sugar like dextrose than with lactose or other of the more complex sugars. This property of alkali formation with the consequent tendency to uncontrolled variation reduces very materially the value of the titer of sugar broths for diagnostic purposes.

The fermentation of dextrose has been determined with every culture by the gas test and therefore this sugar has been considered positive even when the final reaction has given no indication of fermentation. Levulose, galactose, and salicin were in nearly every case fermented at about the same rate as dextrose and consequently were of little value in differentiating one culture from another.

Gas formation.—While we have used the ordinary methods for the foregoing cultural tests, in the study of gas production we have endeavored to find whatever value exact methods may reveal. Our investigations have been concerned with three points of deep interest: the value of exact methods in gas analysis for diagnosis, the constancy of the gas-producing powers of our cultures, and the mechanism of the gas production. Upon this last problem our first series of experiments are not yet complete. The constancy of the physiological characteristics of the bacteria with which we are dealing is a subject which requires not only a preliminary survey but time for the attainment of rigid conclusions, and we shall, therefore, discuss it only incidentally pending more extensive research. Upon the value of exact methods of gas analysis for diagnostic purposes we feel that the preliminary survey we have made has furnished results worthy of publication, and it is with this phase of our researches that we now have to deal.

The ordinary routine methods used in study of the gas production by bacteria are unsuited for quantitative work. The limitations of the Smith fermentation tube were pointed out by the originator and more recently elaborated by Keyes and by Burri and Düggeli.

The chief objections may be summarized as follows:

1. Owing to the solubility of carbon dioxid a large percentage of that product is retained by the medium, and, consequently, an analysis of the supernatant gas does not give a true indication of the volume of gas actually liberated in the fermentation, nor a true ratio between the carbon dioxid and other gases.

2. The medium is exposed to an atmosphere of high carbon dioxid tension in the closed arm and an atmosphere of low carbon dioxid tension over the open arm. Because of this and the high solubility of carbon dioxid in the medium separating the two atmospheres this gas diffuses into the open arm and is lost.

3. The closed arm is anaerobic, the open arm aerobic and the volume of medium exposed to each, as well as the volume of medium furnishing gas to the closed arm, is constantly changing.

One or more of these objections apply to almost all of the methods which are commonly used. The first criticism based upon the solubility of carbon dioxid is perhaps the most widely applicable.

It applies among others to the methods of Escherich, Hesse, Gärtner, Bennett and Pammel, Pakes and Jollyman, Schittenhelm and Schröter, Salus, Fuhrman, and finally that of Harden, Thompson, and Young if used without the corrections they mention. The U-tube of Dunbar was of course practically the same as the device introduced by Smith in 1890. Modifications of the Smith tube with little to recommend them but the ease with which the gas may be drawn for analyses are found in the devices of Ampola and Garino, Pennington and Küsel, Hofstädter, Silberger, Beijerinck and Minkmann, and McCrudden. In addition to these are manometric methods for estimating simply the total gas, as that of Söhle.

Not all of these were employed in studying the gases produced by *B. coli;* but, if we assemble some of the numerous analyses[1] of the gas produced by this extensively studied bacillus, and compare them with analyses made by exact methods, the extent of the error under consideration will become apparent.

To describe in detail each of the various methods employed would be tedious; and while it might be of value, the comparison can be done more thoroughly and justly if the reader has before him the original papers with all the details including the evidence that the bacterium under consideration is a true colon. It will serve the purpose if we simply sketch the results of a few of the many researches.

[1] In the analyses which are to be quoted as well as in those which we shall have to present we shall depart from the custom of expressing the ratio as H_2/CO_2. Instead we shall use the inverted ratio CO_2/H_2. The reason for so doing is twofold. In the first place, we have found in our own analyses that, almost without exception, the volume of carbon dioxid exceeds that of hydrogen. Consequently the ratio CO_2/H_2 is the more convenient one to handle. In the second place, this ratio is more easily converted into the fractional values used rather generally. Thus the ratios $\frac{CO_2}{H_2} = 2.33,\ 1.20,\ 1.00$, may easily be read as $\frac{H_2}{CO_2} = \frac{1}{2.33}, \frac{1}{1.20}, \frac{1}{1}$. Such expressions containing a uniform numerator are more easily intercompared, than the equivalent 3/7, 5/11, 1/1. Furthermore, the ratio CO_2/H_2 has priority to H_2/CO_2 in that it was used by Escherich in 1886. Attention should also be called to the use of the molecular formula of hydrogen, H_2. Various authors, including Escherich and Smith, employ merely the symbol H, a usage very confusing to a chemist, since the data are in terms of hydrogen volumes and should be properly designated as H_2.

Escherich is quoted by Scruel to the effect that the gas produced by *B. coli* is composed of hydrogen, carbon dioxid, and methane. Methane, however, seems to have been mentioned by Escherich only as a constituent of intestinal gas. Escherich found a ratio of CO_2/H_2 approximately equal to 1 for the gases produced by his *B. coli communis* when grown in the presence of $CaCO_3$. This ratio was also found by Chantemesse and Widal and rather widely quoted. But this was criticized by Dunbar on the basis that considerable carbon dioxid must have been liberated from the action of the acids formed upon the calcium carbonate present in the medium used by Chantemesse and Widal. The same criticism applies to Fremlin's experiment in which his medium contained 3 per cent soda.

In Harden's study of the fermentation of glucose by *B. coli* he used in his medium an excess of $CaCO_3$; but he made an attempt to correct not only for the carbon dioxid liberated from this by the acids formed, but also for carbon dioxid retained in solution. With these corrections his data furnished ratios approximating unity. His estimation of the carbon dioxid retained in solution was more or less inaccurate because he failed to consider that there were present all the materials for the carbamino reaction. Upon precipitating (Siegfried) the dissolved carbon dioxid with baryta water it is probable that some carbon dioxid remained in solution associated with the amino bodies and alkalin earths present. If so, the estimate of the CO_2 in solution was too low and the true ratio of CO_2/H_2 was probably greater than 1. Keyes calls attention to the fact that Harden's estimate of the CO_2 dissolved in one instance cannot be applied justly to other cases, since the partial pressure of CO_2 above the liquid varied in each case. To this we may add that reactions taking place in a medium kept neutral with $CaCO_3$ may be very different from those occurring with progressive changes in reaction.

That the ratio is CO_2/H_2 equals 1/1 for the gases *produced* by *B. coli* from dextrose seems, therefore, to have been the fortunate conclusion of several ill-considered investigations. It proved to be fortunate because of the claim of Scruel that the gases were liberated by the decomposition of the formic acid formed in the fermentation, a theory which became the inspiration of a number of profitable researches. These and the one-to-one ratio which Scruel's equation, $CH_2O_2=CO_2+H_2$, would demand of the gases produced by *B. coli* have been lost sight of to a large extent in the numerous investigations on the gas production of bacteria which were made for diagnostic purposes. In these investigations the starting-point was the classic work of Theobald Smith, and the methods were designed with the idea of obtaining results not of analytical accuracy but of comparative value.

With the fermentation tube which he introduced Smith obtained from the action of *B. coli* on dextrose $CO_2/H_2=\frac{1}{2}$. The results obtained by numerous investigators, among whom may be mentioned Pennington and Küsel, Bennet and Pammel, and Mendel, are essentially the same as those found by Smith, and are characterized by the excess of hydrogen over carbon dioxid, owing to the fact that the methods made no provision for the collection of the carbon dioxid held in solution.

Smith's analyses show $CO_2=31.5$–37.2 per cent, $H_2=62.8$–68.5 per cent. Lembke concluded that such results were not reproducible. He found:

6	times	CO_2/H_2	$=1/7$
2	"	"	$=1/6$
4	"	"	$=1/5$
4	"	"	$=1/4$
4	"	"	$=1/2$

Wolffin found $CO_2 = 22$ per cent, $H_2 = 75.6$ per cent, $N = 2.1$ per cent. Stamm contended that 72 hours' growth was necessary to obtain consistent results and published an analysis showing $CO_2 = 31.37$, $H_2 = 58.8$ per cent. Without strict anaerobiosis Pennington and Küsel found an average of 2.09 per cent methane,[1] yet with strict anaerobiosis they could discover no methane in the gas, but found an average of 32.4 per cent CO_2; 64.2 per cent H_2; 3.4 per cent N_2. Bennet and Pammel found 32 per cent CO_2; 68.0 per cent H_2. Fuhrmann found 30.84 per cent CO_2, 62.86 per cent H_2, 6.5 per cent residue; and Pammel and Pammel, 24 per cent CO_2, 75 per cent H_2.

Altho the variation in individual analysis is rather wide, essentially the same results are mentioned by numerous other authors who give merely the ratio $CO_2/H_2 = \frac{1}{2}$.

Among these analyses there is a greater degree of uniformity than Keyes seems inclined to admit. Indeed the comparisons in Keyes's table are not at all happily made, for there are included with the comparable results of Smith, and Pammel and Pammel those of Grimbert, whose medium contained nitrate; the analysis of Schittenhelm and Schroter,[2] who were working with nucleic acids, and the work of Harden who placed his analysis beyond comparison with the others by correcting for dissolved CO_2.

Nevertheless, it takes but a glance at the numerous published analyses of the gas produced by *B. coli* to make one realize that such agreement as exists is attained by the selection of typical analyses, and that Keyes's chief contention, that there are hopelessly wide variations due to inaccuracies of method, is all too true. Perhaps no better illustration is to be found than in the statistical treatment which Longley and Baton have made of a large number of analyses by themselves and others. The statistics presented by Longley and Baton, which are typical of those in other investigations carried out under similar conditions and which indicate "that the quantity of gas formed in confirmatory fermentation tubes varies within wide limits," makes one sympathize with their statement that, "generally speaking, all the evidence we have seems to indicate that there is no determinative significance attached to the quantities of gas and carbon dioxid in the confirmatory or subculture fermentation tube." Even when these authors attempted to overcome the error due to absorption of carbon dioxid in the medium by first saturating the medium with this gas, they obtained, instead of the typical fermentation tube ratio for *B. coli* of $CO_2/H_2 = \frac{1}{2}$, the ratio 3/1, a value very far from that obtained in the more exact investigations, which we have to record, or which Harden or Keyes have presented.

The conclusions of Longley and Baton, as well as those of Howe, to the effect that the gas volume and ratio are in a quantitative sense not reliable for diagnostic purposes, is a conclusion which, we shall see, our results strikingly disprove.

Determination of gas formation by our cultures, made by the usual methods, gave very discordant results. Were we to seek correlations from these data we should encounter the same difficulties met by Longley and Baton, and by Howe.

In view of the difficulties which many workers seem to encounter in obtaining consistent data with the Smith fermentation tube, it is perhaps not out of place for us to suggest that workers must agree upon a more uniform procedure. Nothing is

[1] We should be grateful if told the authority for the statement recurring in textbooks that Klein found the combustible gas to be methane.

[2] Compare Oppenheimer, *Zeit. f. Physiol. Chem.*, 1904, 41, p. 3.

to be gained by the addition of new methods like that of Burri and Düggel , which is similar to that of Seiffert and which has nothing to commend it in the way of accuracy. Nor is there anything to be gained by cumbersome apparatus such as that of Robin or that of Epstein, which retain the gravest fault of the Smith tube.

The chief improvement must involve some means of recovering the gas held in solution by the medium. Two obvious methods for collecting this dissolved gas are: (1) to sweep it out with a neutral gas aided by high temperature if necessary and collect it in a suitable train of absorbents; or (2) to pump it out and analyze it by the ordinary gasometric procedure.

The first method has been elaborated by Stocklasa in his study of nitrogen assimilation. Duchácèk used it in studying the CO_2 output of the typhoid and colon bacteria. The difficulties attending a very accurate determination of hydrogen in such a procedure combined with the necessity for employing rather large quantities of medium make this method objectionable for the study of hydrogen-producing bacteria or for investigations involving the use of expensive material.

For the collection of gas produced by bacteria the mercury pump has been used by Godlewskiego in the study of nitrifying bacteria; by Oppenheimer in some work on intestinal gases; and by Keyes for collecting the gases of *B. coli*. Keyes's paper and his reasons for replacing the Smith tube have already been referred to.

The method we have used is essentially that of Keyes. Several modifications, however, have been made, which we have found better adapted to our work, and which we will describe in detail.

DESCRIPTION OF THE APPARATUS.

The culture bulb used by Keyes we have replaced by a less expensive and more trustworthy form. This is shown in Figs. 1 and 2. It bears a resemblance to one of Roux's Pasteur tubes. The side arm serves for the introduction of the medium and for its inoculation, after which it is sealed off. The other constricted tube is used for the exhaustion of air, after which it also is sealed off. During the period of incubation the bulb is infallibly sealed, and may be left indefinitely without any of the suspicions which stopcocks arouse. After use these bulbs may easily be repaired by anyone having ordinary skill in glass-blowing.

For the first exhaustion we have used the familiar Boltwood mercury pump.

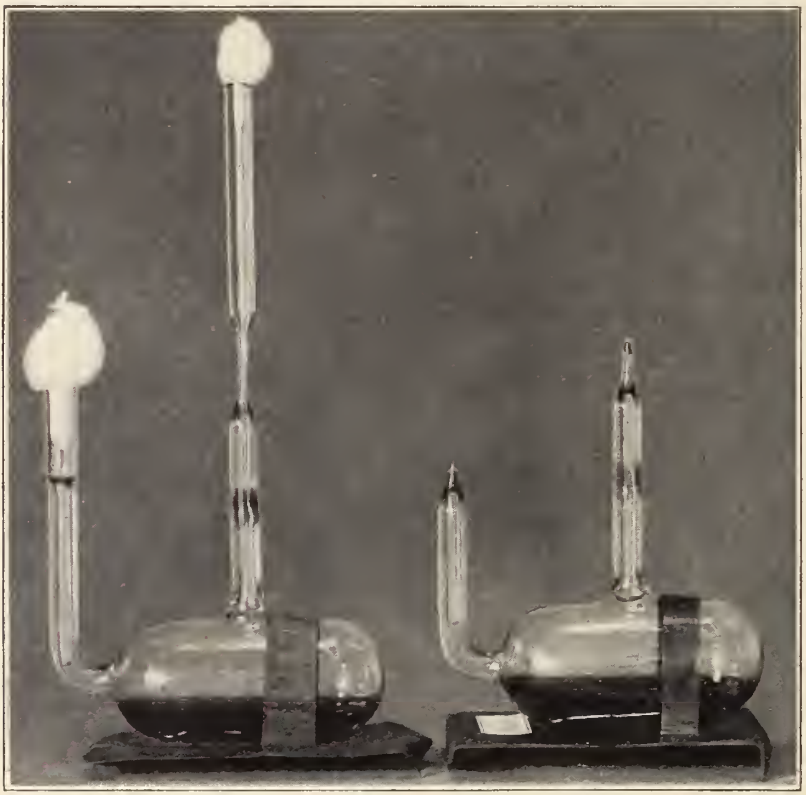
For the collection of the gas we have used the Antropoff modification of the Toepler pump, illustrated in Fig. 3. The essential modification is the inclination of the chamber *A* which prevents in a very effective way the disastrous pounding of the mercury as the gas bubbles through it. By inclining the chamber the gas passes in between the mercury and the upper wall with remarkable smoothness so that a full stroke may be taken at any time without danger of smashing the pump. Two of these pumps which we made in our own laboratory have worked so smoothly that this opportunity is taken to add our recommendation to the many which Dr. Antropoff's simple modification has received.

During the preliminary exhaustion of the bulb with the Boltwood pump the state of the vacuum was estimated by the nature of an electric discharge through a Plücker tube. The evacuation was continued, until, with the connecting stopcock open, the indication of a high vacuum heard in the characteristic sharp click of the mercury in the fall tube was confirmed by the absence of the discharge in the Plücker tube. It is

estimated that the pressure of the gas exclusive of water vapor left in the bulb was well below 0.005 mm.

For estimating the vacuum during the collection of the gas with the Antropoff pump a McLeod gauge was used.

To connect the bulb with the Boltwood pump the straight upright tube of the bulb is slipped through a perforated rubber nipple such as is used for nursing bottles, and is pushed into a short piece of rubber tubing, tied to the inlet of the pump, until



Figs. 1 and 2.—Fermentation bulbs.

the ends of the glass tubes are squarely met. The rubber tube is then securely tied with shoemaker's waxed thread. Now the rubber nipple is brought up till it covers the connection and it is then filled with mercury. This provides a mercury-sealed joint which is both simple and effective. This same form of seal is used in making the connection with the Antropoff pump but the inlet of this is modified. To collect the gas the sealed tip of the bulb (Fig. 2) has to be broken, and in order that this may be accomplished the inlet was so blown that it might receive the taper of the seal as shown in detail, Fig. 4. At the point *e* the tube was collapsed to an elliptical cross-section,

so that the tip of the seal when broken off cannot jam; *f* is a piece of stout rubber tubing tied with waxed thread, and *g* a rubber nipple filled with mercury.

Above the connection in each pump is a mercury-sealed stopcock. By opening this only occasionally there is prevented a continuous distillation of water from the medium into the drying agent.

The drying tubes of both pumps are for sulfuric acid. While sulfuric acid, if given time and proper circulation, is quite as good a drying agent as phosphorus pentoxid, it is of course not so efficient for rapid drying. The small difference, however, is compensated for by the greater ease with which sulfuric acid may be renewed, a factor which is of considerable importance when a large number of pumpings are to be made, and when none of these need to be the extremely high vacuum necessary in other classes of work.

The form of drying tubes shown in Fig. 3 has proved very satisfactory. The acid is poured in at *a* until it fills the tube some distance above the horizontal body *b*. Upon draining the acid out through *c* the walls are left "moistened." It is only drained, however, until there is a layer half filling the body, and there is left a clear space above for the flow of gas. The stopcocks *a*, *e*, and *f* are mercury-sealed, and by leaving a few drops of acid in *a* and keeping the tube *c* filled by trapping the acid with a beaker, as shown in the figure, the cocks are made perfectly gas-tight.

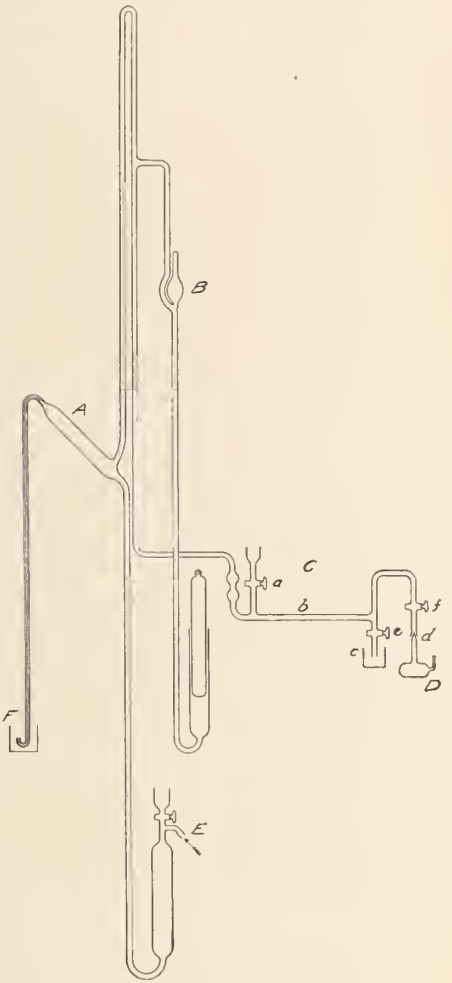


FIG. 3.—Vacuum pump and connections.

The initial action of the acid on the stopcock lubricant is troublesome but this soon disappears, and if care be taken to avoid jamming the cocks in the effort to make them snug the lubricant will last for months. This form of drying tube has proved to be capable of some very nice adjustments. By providing the bulbs as shown, the tube may be filled with the acid sufficiently to force the gas to bubble through it, thereby giving it additional efficiency in a preliminary drying of the pump. During an evacuation, of course, a free passage must be allowed for the gas, but the leveling of the tube may be easily adjusted, so that if too rapid a current of gas is accidentally allowed to enter, it will cause a wave of acid to be quickly formed. This, closing across the tube, requires the gas to bubble through it. Then, when the current of gas has subsided, the acid returns to its normal level, leaving

a clear passage above. It is believed that more efficient drying is procured by having the entering gas impinge directly upon the layer of acid. If during the evacuation of a large body of medium it should be thought desirable to renew the acid, the pump may be stopped and brought to atmospheric pressure and the acid renewed. The pump is then re-evacuated without the vacuum already created in the vessel being exhausted, provided the cock between the vessel and the pump is kept closed.

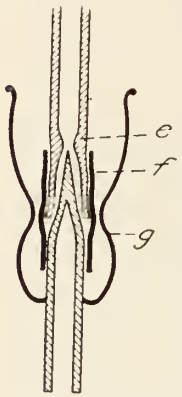


FIG. 4.—Method of joining bulb to pump.

The detailed procedure is as follows: With the open arms of the fermentation bulb plugged with cotton the clean dry bulb is sterilized one hour at 170° C. (dry heat). Five cubic centimeters of medium are then run in from a pipette through the side arm. The bulb is of such capacity that these 5 c.c. lie well below the inlet of the side arm. After a period of three intermittent sterilizations in the Arnold, the bulb is ready for inoculation. This is easily made by tipping until the medium runs into the cup of the side arm and there it is inoculated with a loopful of the culture to be studied. After the inoculated liquid has been allowed to drain back into the bulb the side arm is fused off in a blast lamp. If care is used to draw the seal off gradually to a point and to avoid too blunt an end, there is little danger of its cracking even if poorly annealed. The next step is the exhaustion. This is done with the Boltwood pump until, as described above, the exhaustion of the bulb is practically complete. The connecting tube is now fused at the constriction in such a way that there is left a long tapering seal. After the period of incubation the gas which has been formed is collected as follows: The tapering seal is first scratched with a diamond and then connection is made with the Antropoff pump, as previously described. The pump is next exhausted and when this is complete to below 0.01 mm. the connecting stopcock is closed and the tip of the seal on the fermentation bulb is broken by a sharp turn. The gas is now allowed to flow slowly into the pump by carefully opening the cock *f*, Fig. 3. The receiver for the gas is illustrated in Fig. 5 and needs little explanation. Before filling it with mercury the interior is moistened with water in order that the gas when collected may become saturated before analysis. To fill the receiver with mercury the air is aspirated from *G* through *H*. This outlet is buried under mercury, so that it is sealed against entrance of air both during collection of gas from the pump, and during transference to the burette through a connecting capillary tube.

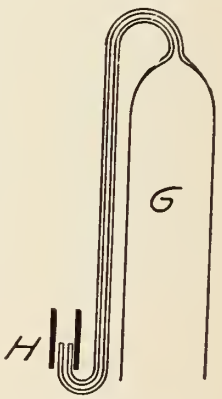


FIG. 5.—Gas receiver.

Just as in the preliminary evacuation with the Boltwood pump, so in the collection of the gas, a stopcock is used to shut off the continual distillation of water. This cock is opened for a moment only after every third or fourth stroke of the pump. The drying tube is like that previously described. It might be thought that the use of sulfuric acid as a drying agent for gas containing a large percentage of carbon dioxid would be objectionable because concentrated sulfuric acid is an even better absorbent of CO_2 than water. But there is no reason to believe that at the vacuum

attained there is any appreciable amount of carbon dioxid retained. Two burettes were used for the analysis, one of 30 c.c. and one of 15 c.c. capacity, both finely calibrated. The Hempel pipettes were of 20–30 c.c. capacity. The device for connecting a burette with a pipette is shown in Fig. 6. Altho it is essentially the same in principle as the device described in Hempel it possesses a distinct advantage for working with small volumes of gas in that there are no additional stopcocks with which the gas may come in contact. The bore of the connecting capillary is but 0.5 mm. and, fortunately, a very neat T-joint was made so that the separation of gas and absorbent at this point could be made with a high degree of accuracy.

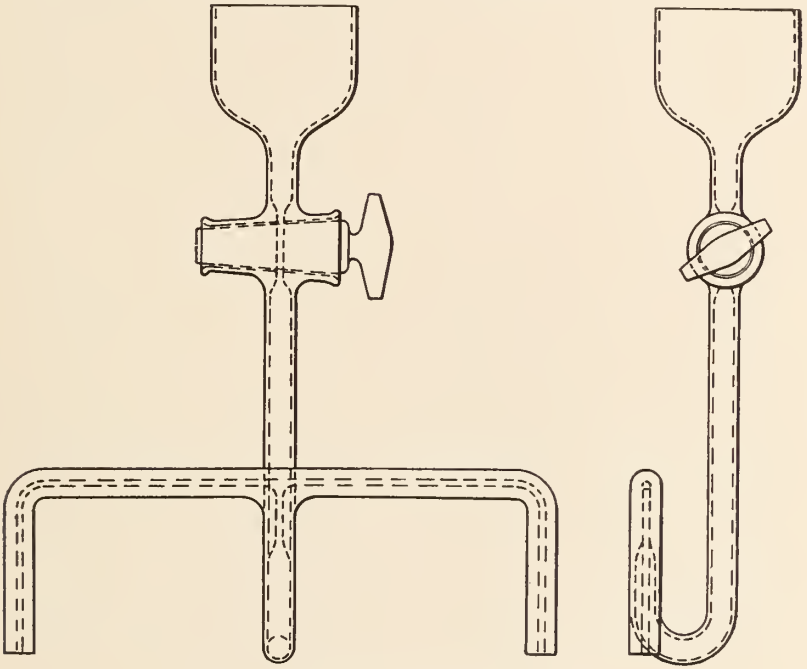


FIG. 6.—Attachment for separating gas from absorbent.

All the analyses were made over mercury. For the estimation of combustible gases, explosion with oxygen was alternated by burning in oxygen with a heated platinum spiral as recommended by Dennis and Hopkins.

Inasmuch as the volume of hydrogen estimated by the contraction after combustion came to within about 0.05 c.c. of the residual gas left after absorption of CO_2, and inasmuch as the gas after combustion showed no further contraction when placed over KOH in the many cases tried, it may reasonably be assumed that the combustible gas was in all cases exclusively hydrogen. Therefore in the majority of analyses, when the calculated hydrogen came within a few hundredths of a cubic centimeter of the gas left after absorption with KOH, it was concluded needless to carry the analysis farther.

In all data presented the gas volumes have been reduced to 760 mm. of mercury and 0° C.

THE CONSTANCY OF RESULTS.

Considering the gas analyzed as simply end products obtained by the specific method outlined, let us see whether the results are reproduced with sufficient constancy to justify their use in diagnosis.

In the first place, duplicate determinations were found to agree, as will be seen by studying Table 9. Thus *fg*, which we received from the Hygienic Laboratory of the Public Health Service and which was designated "*B. coli communis*," gave the following:

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
(4.14	2.22	1.88	53.6	45.4	1.18)
7.50	3.93	3.54	52.4	47.2	1.10
8.27	4.34	3.93	52.5	47.4	1.10
8.72	4.57	4.15	52.4	47.6	1.10

ay gave:

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
8.06	4.40	3.64	54.6	45.2	1.21
8.40	4.60	3.81	54.4	45.0	1.21

As the work progressed it was found that the determinations fell into certain groups and that there was a marked agreement in the data of different organisms. As an example, *bs*, *bn*, *cg*, and *ct* all gave about the same amount of gas and the identical ratio 2.01. When this occurred, it was considered safe to regard these organisms as identical so far as the gas-producing powers were concerned, and duplicate determinations were omitted because of the large amount of work entailed. It is only fair to point out that this failure to make duplicate determinations in favorable cases, and the repetition of our less harmonious results, all of which we have carefully included in the tables, make the inconsistencies appear greater than they probably should have been.

Altho close agreement of duplicate determinations made at the same time indicate the accuracy of the method, it may still be true that the physiological activity of an organism may vary with successive transfers.

Toward the end of the series we repeated several determinations, which were found to be in poor agreement among themselves or with the groups to which they seemed to belong. We thus

obtained determinations between which a considerable time elapsed. But inasmuch as only the apparently abnormal cases were chosen for repetition, the comparisons are, strictly speaking, not a fair test of the issue. One case demands special notice: *cw*, in November, 1911, gave:

No. of Trans.	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c.	%	%	
12	13.61	9.51	4.10	69.9	30.1	2.31

Six months later, after 6 more transfers, *cw* gave:

No. of Trans.	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c	%	%	
18	7.21	3.76	3.36	52.2	46.6	1.12
	7.02	3.69	3.29	52.6	46.9	1.12
	7.45	3.87	3.43	51.9	46.0	1.13

It was thought at first that this might be a case of a change in gas-forming power. A dried culture of the eighth transfer, made 4 months before the ratio 2.31 was obtained, was then plated out, and with this new culture the following determinations were made:

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
7.83	4.16	3.64	53.2	46.5	1.20
7.73	3.99	3.55	51.6	46.0	1.12

Thus the eighth and eighteenth transfers, 10 months apart, are identical in gas-producing power. What the cause of the singularity in the twelfth was is not known. It is not unlikely that in making the inoculation *cv* (203–204)[1] was mistaken for *cw*, since the two agree both in total volume and ratio, and since the tube of *cv* standing next to *cw* in the rack might have been picked up by mistake.

In Table 3 are the other comparisons. The numbers in the first column are the reference numbers of Table 9. The number of the transfer from which the inoculation was made is in the second column. Since one transfer was made each month, the time elapsing between the determinations can be seen at a glance.

[1] Figures are the reference numbers in Table 9.

TABLE 3.

GAS PRODUCTION BY CULTURES AT DIFFERENT TIMES.

5 c.c. of Standard Broth Containing 1 Per Cent Dextrose, Incubated 7 Days at 30° C.

Reference No.	No. of Transfer	Designation of Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio
			c.c.	c.c.	c.c.	%	%	
6	6	em	7.24	3.69	3.54	51.0	48.9	1.04
7	6	em	7.65	3.98	3.67	52.0	48.0	1.08
8	7	em	0.81	0.44	0.26	54.3	32.1	1.69*
9	11	em	9.44	4.80	4.67	50.9	49.5	1.03
83	9	bc	8.02	4.43	3.56	55.3	44.4	1.24
84	9	bc	2.09	1.10	0.96	53.1	45.9	1.15
85	15	bc	7.83	4.22	3.58	53.9	45.7	1.18
55	22	al	8.97	4.63	4.36	52.0	48.9	1.06
56	22	al	9.62	5.22	4.37	54.3	45.4	1.19
57	31	al	7.56	4.08	3.47	54.0	45.9	1.18
73	11	db	8.18	4.39	3.78	53.7	46.2	1.16
74	17	db	7.63	4.09	3.49	53.6	45.7	1.17
118	14	cz	14.93	9.03	5.91	60.4	39.6	1.53
119	17	cz	12.54	7.59	4.95	60.5	39.5	1.53
120	17	cz	12.28	7.60	4.68	61.8	38.2	1.62
138	12	cs	17.62	11.39	6.26	64.6	35.5	1.82
139	19	cs	16.03	10.34	5.69	64.5	35.5	1.82
141	12	cy	17.39	11.46	5.90	65.9	33.9	1.94
142	17	cy	0.05					
143	19	cy	15.31	9.67	5.59	63.2	36.5	1.73
147	11	ao	16.46	11.95	4.45	72.6	27.0	2.69
148	11	ao	16.96	9.22	4.72	54.4	27.8	1.95
149	20	ao	14.79	9.14	5.61	61.8	37.9	1.63
150	21	ao	14.18	8.78	5.34	61.9	37.7	1.64
151	21	ao	14.78	9.22	5.47	62.4	37.0	1.69
154	23	n	12.19	8.10	4.10	66.4	33.6	1.98
155	23	n	10.62	6.66	3.95	62.7	37.2	1.69
156	33	n	13.94	9.48	4.41	68.0	31.6	2.15
158	13	cq	17.66	11.70	5.96	66.3	33.8	1.96
159	20	cq	14.41	9.50	4.89	65.9	33.9	1.94
160	12	cr	17.68	11.51	6.16	65.1	34.8	1.87
161	19	cr	14.55	9.84	4.69	67.6	32.2	2.10
166	12	bj	no	growth				
167	15	bj	2.56	2.21	0.31	86.3	12.1	7.13
168	16	bj	13.44	9.05	4.38	67.3	32.6	2.07
169	17	bj	12.50	8.40	4.17	67.2	33.4	2.01
173	3	ev	12.90	8.62	4.16	66.8	32.3	2.07
172	3	ev	13.11	8.84	3.93	67.4	30.0	2.25
174	10	ev	12.19	8.12	4.04	66.6	33.1	2.01
211	57	w	22.41	15.90	6.54	71.0	29.2	2.43
212	57	w	13.62	9.56	3.14	69.9	23.1	3.03
213	57	w	21.61	15.80	5.52	73.1	25.5	2.86
214	57	w	13.06	9.29	3.83	71.1	29.3	2.43
218	14	ce	1.07					
219	16	ce	2.32	1.89	0.40	81.5	17.2	4.12
220	17	ce	3.54	2.75	0.76	77.7	21.5	3.62
221	8	fb	2.28	2.21		97.0		
222	13	fb	4.02	4.01		99.9		
223	13	fb	1.39	1.39		100.0		

* Notice the small volume of gas which may account for this high ratio.

THE GASES ANALYZED ARE ONLY END PRODUCTS.

In estimating the value of the analyses made in accordance with the method we have outlined, it must be remembered that the gases recovered may not necessarily furnish exact data on the true amounts of the gases evolved by the principal reactions of the fermentation. Secondary reactions may enter in to alter the relative quantities of the constituent gases. Harden, for instance, observed that when *B. coli* is cultivated in a medium containing asparagin, the asparagin is reduced to ammonium succinate, presumably by the hydrogen, since the volume of hydrogen recovered is correspondingly diminished. In view of this it would seem highly probable that the low percentage of hydrogen which Keyes found for *B. coli*, grown on Dolt's asparagin medium, was due to oxidation of a portion of the hydrogen by the asparagin. This seems the more probable because with a medium containing no asparagin Keyes obtained a much higher percentage of hydrogen, with asparagin medium, 63.27 per cent of CO_2 and 36.05 per cent of H_2; with broth, 55.73 per cent of CO_2 and 43.56 per cent of H_2. We have obtained similar results.

TABLE 4.

INFLUENCE OF COMPOSITION OF MEDIUM ON GAS PRODUCTION OF *fg*.

5 c.c. of Medium Incubated 7 Days at 30° C.

Vacuum Apparatus.

Medium	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c.	%	%	
Standard dextrose broth	4.14	2.22	1.88	53.6	45.4	1.18
	7.50	3.93	3.54	53.4	47.2	1.10
	8.27	4.34	3.93	52.5	47.5	1.10
	8.72	4.57	4.15	52.4	47.6	1.10
Standard dextrose broth + 0.1% asparagin	7.13	4.22	2.88	59.2	40.4	1.47
1% dextrose, 0.1% asparagin, 0.5% K_2HPO_4	4.89	2.99	1.86	61.1	36.1	1.61

But if we admit oxidation of the hydrogen to be a factor in lowering the amount set free from a medium containing asparagin, we must concede the possibility of oxidation of the nascent hydrogen occurring in a medium as complex as broth. There is no doubt that reductions by growing cultures of *B. coli* and other bacteria are powerful. Escherich and Pfaundler, Emmerich, Leiningen, Grafu and Loew have even observed a reduction of iron oxid

which they attributed to the nascent hydrogen liberated in a butyric fermentation. We must therefore keep it clearly in mind that the hydrogen recovered is the quantity found as an end product.

In almost all of the analyses we have made, there is to be found a small but appreciable amount of "residual gas" which we are fully justified in calling nitrogen. This finding is in accord with all the other analyses which we have been able to discover in the literature. The large percentages frequently recorded have no doubt been largely due to the fact that no attempts were made to remove the air from the medium previous to the growth of the organisms. While fully recognizing the difficulty with which last traces of nitrogen may be removed from any liquid even on boiling, we judge that the quantity of nitrogen found is in excess of the amount which may be accounted for by that source. The average amount found for the colon group is about 0.05 c.c. or about 0.7 per cent of the total gas, which is in close agreement with the analyses of Keyes. It is known that hydrogen-producing bacteria are capable of reducing nitrates with the liberation of free nitrogen. It is therefore not improbable that some, at least, of the nitrogen observed in the gas collected originated in the trace of nitrates or nitrites which existed either as original impurity of the materials (Wherry) used in the medium or which gained entrance from the laboratory atmosphere.

On the other hand we have found about this same amount of "nitrogen" in the analysis made of the gas produced by certain bacteria which furnish no hydrogen. Here there still remains the possibility of reduction of nitrates by other agents.

Some such origin of the nitrogen appears to be more plausible than incomplete removal of atmospheric nitrogen inasmuch as no detectable amount of oxygen has been found associated with it even in cases where there was no bacterial growth.

The question of whether any CO_2 is left unremoved after the preliminary evacuation and then liberated or held back by the media when the gas is collected cannot be adequately answered.

There are but few data on the retention of gases by liquids under high vacuum, and while it is true that numerous investigations are recorded on the solubilities of carbon dioxid in water, in solution of

salts, in colloidal solutions and suspensions, and in certain specific mixtures such as blood and beer, it would be unsafe to transpose the data of any of these to a medium as complex as broth, in which the bacteria are producing not only a profound chemical change, but distinct physical alterations.

It is true that we have used in the majority of cases a medium which not only contains dibasic potassium phosphate (K_2HPO_4) but which was neutralized with sodium hydrate. It is absolutely impossible to predict the exact amount of CO_2 which would have been absorbed by this medium and retained after the first evacuation. Furthermore, determinations of this quantity in one case would not necessarily hold for another. We have therefore preferred to let this error go uncorrected, having satisfied ourselves that it is far too small to affect in any noticeable degree the relative values we have used in making correlations, and too small to make very much difference in the absolute values of the ratios.

It must not be understood, however, that this small possible error has escaped our attention. We will deal with it more fully in the presentation of other results we hope soon to publish. We have considered it advisable to neglect minor sources of error until the preliminary survey is made. Then, with fuller knowledge of the large problems, we may improve methods in the direction required.

It is found that while single determinations may sometimes vary from the mode, the averages tend to place the organism definitely in a group. The significance of the grouping is more plainly seen by plotting the values of Table 9 (for the conditions under which these values were obtained see p. 442). This plot is given in Fig. 7 in which the abscissae represent volumes of carbon dioxid and ordinates volumes of hydrogen. The group about $x=4.3$ $y=3.6$ we shall call Group I; that between $x=6$ and $x=12$, Group II, and those points beyond $x=12$, Group III. In the case of those four single determinations which seemed to constitute a new group about the point $CO_2=11.5$, $H_2=6.0$ (see Fig. 7), and which happened to have been made with the same batch of medium, new values all fell within the most thickly dotted section of Group II.

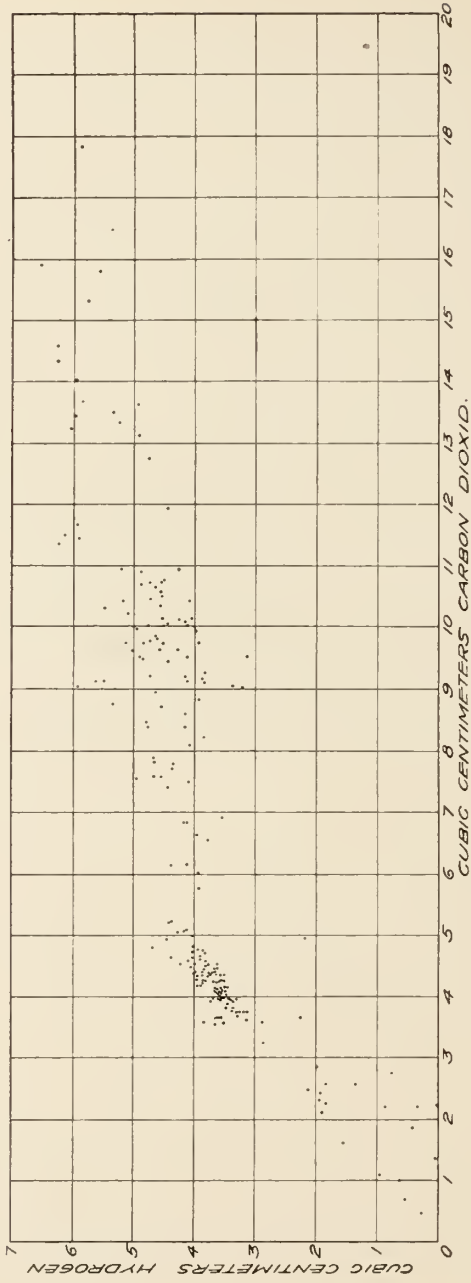


Fig. 7.—Relation of carbon dioxid to hydrogen as shown in Table 9.

Discrepancies do occur as in the comparison of *ao* and *aj*. Thus *ao* of Group II gave in the fourth determination values similar to those of *aj* of Group III, and *aj* in the sixth determination gave values easily confused with those of *ao*.

TABLE 5.

COMPARISON OF THE GAS PRODUCTION OF *ao* AND *aj*.

Organism and Reference Nos.*	Experiment Number	Total Volume	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
		c.c.	c.c.	c.c	%	%	
ao 147–151	1	14.78	9.22	5.47	62.4	37.0	1.69
	2	14.18	8.78	5.34	61.9	37.7	1.64
	3	14.79	9.14	5.61	61.8	37.9	1.63
	4	16.46	11.95	4.45	72.6	27.0	2.69
	5	16.96	9.22	4.72	54.4	27.8	1.95
aj 191–196	1	20.48	14.06	5.97	68.7	29.1	2.36
	2	19.51	13.47	5.96	69.0	30.6	2.26
	3	21.08	14.60	6.23	69.3	29.6	2.34
	4	19.38	13.25	6.04	68.4	31.2	2.19
	5	23.88	17.85	5.87	74.7	24.6	3.08
	6	14.04	9.76	5.11	65.3	34.2	1.91

*See Table 9.

Similarly, two determinations with *w* (Table 9), fall in Group II altho their ratios and the complete returns in two other cases place *w* clearly in Group III.

In the dense and well consolidated Group I, the most uniform results were obtained; but it was also the members of this group which contributed most to the area of scattered dots in the plot between I and the origin. For instance *em*, a colon obtained originally from Kral, gave in three analyses 6, 7, and 8, values which fell within the Group I, while in a fourth, Experiment 9, it gave only 0.81 c.c. of gas. A similar but less prominent case is that of *fg*, 30–33, a colon which we have studied rather extensively. The tendency among them to adhere to the $x=y$ line, together with the fact that the long axis of Group I has the slope of the $x=y$ line, would confirm the opinion that the low values are simply those of inhibited cultures which, if unobstructed, would have reached the values of Group I along the same route.

The exact nature of this inhibition is not known. It is a well known fact that cultures kept in a laboratory for a long time frequently lose something of their ability to ferment; but to ascribe this to an "attenuation" is merely renaming the phenomenon. Is

it comparable to those limitations of "diet" such as in the case of higher animals inhibit growth while allowing maintenance (Osborne and Mendel), or is it due to the natural selection of those individuals which are capable of thriving with the least energy requirements that laboratory conditions and media demand?

Undoubtedly, in certain instances negative tests result from poor inoculation. We have found rare instances where this was supposed to have happened, as in the case of *cy* (142) *bj* (166) and *by* (117). Dr. Erwin Smith suggests that the occasional failure of an organism to produce fermentation may be due to a scanty seeding; the reason being that, if a medium is unfavorable to growth, there must be a sufficient number of bacteria introduced to combat the initial toxicity. This he calls the "mass action" of bacteria.

The exact cause of the apparent "attenuations" is not known, and we have considered it best to include all these cases, since the presentation of all instances gives a fairer picture of the actual value of the data.

VARIATION DUE TO THE DIFFERENT CONDITIONS.

Attention has already been called to the variation in the ratio CO_2/H_2 for *B. coli* when grown in different media. The case mentioned was explained on the assumption that some of the hydrogen was oxidized. On the other hand, Mendel observed a marked change in ratio with increase in the concentration of sugar. With the higher concentrations less gas was obtained and with decrease in total gas, Mendel observed a decrease in percentage of carbon dioxid. In Table 6 are comparisons between Mendel's determinations and those we have made.

The change noted by Mendel is easily explained by the fact that he drew for analysis only the gas collected above the medium. Consequently when the total volume of gas was small, as both observers have found with high concentrations of sugar, a larger percentage of carbon dioxid remained dissolved than when the total volume of gas was large, and this dissolved carbon dioxid Mendel neither collected nor corrected for.

As the table will show, we have been unable to confirm the change in ratio noted by Mendel. Indeed, we have found that

when the ratio of carbon dioxid to hydrogen is in the neighborhood of 1.1–1.2 for dextrose broth, this ratio is constant under many varying conditions. On the other hand, the higher ratios are more likely to vary.

Thus while the ratio for *fg* varied but slightly with change in the concentration of the sugar, Table 7 will show considerable variation in the ratio of *aj*.

TABLE 6.

INFLUENCE OF SUGAR CONCENTRATION UPON GAS PRODUCTION BY *B. Coli Communis*.

DEXTROSE CONTENT	AFTER MENDEL				BY PUMPING (5 C.C. MEDIA USED)			
	Total Gas	CO_2	H_2	Ratio CO_2/H_2	Total Gas	CO_2	H_2	Ratio CO_2/H_2
%	c.c.	%	%		c.c.	%	%	
0.5	84.7	31.25	67.23	0.46	7.60	53.80	43.15	1.17
1.0	83.9	30.39	68.58	0.44	7.16	52.70	46.90	1.12
3.0	84.2	30.28	68.43	0.44	5.20	52.75	45.90	1.15
6.0	90.4	28.65	70.39	0.41	4.71	54.15	45.45	1.19
10.0	51.9	26.58	72.66	0.36	5.30	54.75	45.55	1.20
18.0	9.7	10.30	89.70	0.11				
20.0					3.75	53.30	44.00	1.21

TABLE 7.

INFLUENCE OF SUGAR CONCENTRATION UPON GAS PRODUCTION BY *aj*.

5 c.c. of Medium Incubated 7 Days at 30° C.

Concentration of Dextrose	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
%	c.c.	c.c.	c.c.	%	%	
½	10.33	6.23	4.08	60.3	39.5	1.53
	9.90	5.65	4.18	57.1	42.2	1.35
1	19.71	13.83	5.66	69.2	29.9	2.33 averages
6	16.62	12.45	4.18	74.9	25.1	2.98
10	15.53	12.00	3.51	77.3	22.6	3.42
	16.43	12.24	3.65	74.5	22.2	3.36
20	14.13	10.35	2.84	73.3	20.1	3.64
	13.10	10.24	2.88	78.2	22.0	3.56

Again, if we compare *fg*, a colon, with *ev* (bacillus of dysentery) and *aj*, we shall find little effect of incubation temperature upon *fg*, but a very noticeable effect upon *aj*.

Attention should be called to the fact that the amount of gas is doubtless an indication of the ability of the organism to grow at certain temperatures and not an influence on the function of gas formation alone. For instance, *aj* has an optimum temperature below 37° C. and grows at this temperature only sparsely.

It might be argued that the 7 days' incubation was insufficient to allow the activities of the organism or their liberated enzymes to

come to their final rest. Were this true, and were cultures to progress unequally, the determinations would have been made at different stages. It was found, however, by following the rate of gas production of *fg*, a colon, Group I, that its activity had almost ceased at the end of 48 hours. A determination made with *a*, of Group II, incubated 54 days, showed a slight increase of total gas over the amounts found at the end of 7 days, but the identical ratio. With *aj* of Group III, cultures incubated for different lengths of time up to 100 days gave more or less irregular results from which no definite conclusions may be drawn other than to say that the 7 days' incubation seemed sufficient, and that no advantage was apparent in longer incubations.

TABLE 8.

Comparison of the Gas Production of *fg*, *ev*, and *aj*, at Different Temperatures.

5 c.c. of Standard Medium Incubated for 7 Days.

Temp. °C.	Symbol of Organism	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
		c.c.	c.c.	c.c.	%	%	
18.....	fg	7.33	3.97	3.30	54.2	45.1	1.20
		8.20	4.45	3.69	54.3	45.1	1.21
30.....	fg	4.14	2.22	1.88	53.6	45.4	1.18
		7.50	3.93	3.54	52.4	47.2	1.10
		8.27	4.34	3.93	52.5	47.5	1.10
		8.72	4.57	4.15	52.4	47.6	1.10
37.5.....	fg	7.42	3.85	3.55	51.9	47.9	1.08
		8.47	4.36	3.99	51.5	47.1	1.09
30.....	ev	13.11	8.84	3.93	67.4	30.0	2.25
		12.90	8.26	4.16	66.8	32.3	2.07
		12.19	8.12	4.04	66.6	33.1	2.01
37.5.....	ev	9.41	6.16	3.25	65.4	34.6	1.89
		11.64	7.11	4.47	61.1	38.4	1.59
18.....	aj	15.14	10.11	5.03	66.8	33.2	2.01
		15.48	10.23	5.26	66.1	34.0	1.94
20.....	aj	20.85	14.45	6.26	69.3	30.0	2.31
30.....	aj	19.71	13.83	5.66	69.2	29.9	2.35 averages
37.5.....	aj	1.15	0.69	0.15	83.2	12.9	6.44
		1.13	0.95	0.13	84.3	11.5	7.36

On the other hand, the slow fermenting power of certain other bacteria such as *fm* (Table 21) has led us carefully to follow the progress of gas production of this organism, and it will be well to do the same with typical members of the groups already described.

The tendency of the colon bacillus to retain its characteristic ratio is exhibited, not only in the cases cited, but also in its action upon different sugars and allied compounds. On the other hand, *aj* has been found to give different ratios not only under different conditions but in its fermentation of different carbohydrates.

Much of the data upon this phase of the subject will be reserved until a projected series of experiments are completed; but attention is now directed to it because this same constancy among the lower ratios and the variability among the higher is seen in the results where uniform conditions were supposed to have obtained. Fig. 7 will show this at a glance: Group I is a well consolidated area; Group III is a sparsely dotted area. Consolidation of this might or might not have been more marked if more values had fallen within it. But Group II cannot be said to lack in number of determinations and yet these are scattered broadly.

A similar constancy in the colon group and variation in the data from organisms producing larger amounts of CO_2 was observed by Russell and Bassett.

We shall develop in a subsequent chapter a hypothesis explaining this discrepancy, but we cannot depend upon speculation when we come to use the data for purposes of correlation. We must see to what extent the greater or less variation indicated in Groups II and III renders the data unavailable.

In answer, it may be said at once that the values for any given organism are very much less variable than those which have been obtained by the older methods, and which have been used in differentiating species. In the second place, the inconstancy in the great majority of cases is within certain well defined limits. Altho there are what we may call transitional values between Groups I and II and between Groups II and III (see Fig. 7), yet these groups are rather clearly defined.

If the analyses are given their face value, and it is held that each of the determinations, or the average of each culture, represents a constant and definite characteristic of the organism concerned, then it will be found that by dividing them into species upon this basis alone there results a graded series of transitional species from one group to another. The logical conclusion would be a multiplication of species beyond the limits of convenience for systematic classification. But even if we admit these species to be real, we are forced to the conclusion that certain well defined characteristics are quantitatively established which throw the present series into groups, and that of these Group I, at least, is unequivo-

cally distinct. It therefore remains to be considered whether the transitional forms are truly such, or are mere suppositions imposed by the errors of the method of study. Enough data have been presented to show that some of the organisms furnish different ratios under different conditions. Until we know more thoroughly the subtle influences surrounding the cultivation of bacteria we cannot indulge in the excuse that failure to obtain *exactly* concordant results is due to variation in what may reasonably be assumed to be fundamental physiological processes. The marked increase in constancy exhibited by the abandonment of inexact for more exact methods justifies the prophecy that further improvements of method will not only reveal the physiological processes to be responsive in like degree to like influences, but that fixed characteristics will be established as a sound basis, not only for diagnosis but for the study of whatever variations or mutations do occur.

While, therefore, strict logic forbids our eliminating the "transitional" values, we feel justified in assuming that our data have indicated certain well defined groups. Using these groups as a basis, let us proceed to find whatever value they may have as a basis for the correlation of other cultural tests.

For the purposes of correlation we have assembled the results of determinations made under the following uniform conditions.

The medium was prepared as follows:

To 1,000 c.c. distilled water were added 4 gm. Liebig's beef extract and 10 gm. Witte's peptone. This was heated on a steam bath for 20 min., filtered, and the loss of water made up.

The acidity of a sample was determined by titration with tenth normal sodium hydrate using phenolphthalein as indicator. The whole was then neutralized with normal sodium hydrate (10 c.c. usually required 0.7 c.c. tenth normal sodium hydrate). After 5 minutes' heating the medium was again filtered, its acidity determined, and if 10 c.c. required more than 0.2 c.c. tenth normal sodium hydrate it was neutralized, heated, and filtered again. There were now added 1 per cent dextrose (Kahlbaum) and 0.5 per cent dibasic potassium phosphate (K_2HPO_4). After solution of these was complete, the medium was filtered and its volume at room temperature made up to 1,000 c.c. It was then tubed and sterilized 3 consecutive days in an Arnold sterilizer.

Exactly 5 c.c. of this medium were used in each determination. The period of incubation was 7 days, and the temperature 30° C. In Table 9 the numbers in the first column are given simply for convenience later in referring to a particular determination. The second column shows the laboratory designations of the organisms. In the three succeeding columns are, respectively, the total volumes, the volumes of

TABLE 9.

GAS PRODUCTION OF BACTERIA GROWN IN VACUUM BULBS.

5 c.c. of Standard Broth Containing 1 Per Cent Dextrose Incubated 7 Days at 30° C.

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
1	h	7.46	3.58	3.84	48.0	51.5	0.93	
2	h	7.32	3.55	3.65	48.5	49.9	0.97	
								0.95
3	f	7.09	3.58	3.50	50.5	49.4	1.02	1.02
4	cf	3.26	1.64	1.59	50.4	48.8	1.03	1.03
5	eq	8.23	3.85	3.72	46.8	45.1	1.04	1.04
6	em	7.24	3.69	3.54	51.0	48.9	1.04	
7	em	7.65	3.98	3.67	52.0	48.0	1.08	
8	em	(0.81	0.44	0.26	54.3	32.1	1.69)*	
9	em	9.44	4.80	4.67	50.9	49.5	1.03	
								1.05
10	bh	7.88	4.10	3.68	52.0	46.7	1.11	
11	bh	7.24	3.64	3.64	50.3	50.3	1.00	
								1.06
12	bg	7.80	4.07	3.64	52.2	46.7	1.12	
13	bg	7.24	3.64	3.60	50.2	49.7	1.01	
								1.07
14	p	8.39	4.39	4.01	53.3	47.8	1.09	
15	p	8.71	4.52	4.22	51.9	48.4	1.07	
								1.08
16	ax	7.37	3.83	3.49	52.0	47.4	1.10	
17	ax	7.74	3.95	3.75	51.0	48.4	1.05	
								1.08
18	bt	8.09	4.21	3.86	52.0	47.7	1.09	1.09
19	bi	5.02	2.58	2.38	51.4	47.4	1.08	
20	bi	6.94	3.65	3.28	52.6	47.3	1.11	
								1.10
21	ds	4.09	2.12	1.93	51.8	47.2	1.10	1.10
22	fp	1.31	0.66	0.60	50.2	45.6	1.10	1.10
23	bf	8.65	4.48	4.06	51.8	46.9	1.10	
24	bf	8.13	4.25	3.81	52.3	46.9	1.12	
								1.11
25	bm	8.44	4.40	4.00	52.1	47.4	1.10	
26	bm	8.31	4.32	3.97	52.0	47.8	1.09	
								1.10
27	ek	7.11	3.77	3.39	53.0	47.7	1.11	1.11
28	aq	9.40	4.94	4.41	52.6	46.9	1.12	1.12
29	aq	(3.54	.....	.....	.....	.....	.....)*	
30	fg	4.14	2.22	1.88	53.6	45.4	1.18	
31	fg	7.50	3.93	3.54	52.4	47.2	1.10	
32	fg	8.27	4.34	3.93	52.5	47.5	1.10	
33	fg	8.72	4.57	4.15	52.4	47.6	1.10	1.12
34	az	7.90	4.22	3.62	53.4	45.8	1.17	
35	az	8.19	4.19	3.96	51.2	48.4	1.08	
								1.12
36	bb	8.04	4.26	3.85	52.7	47.6	1.11	
37	bb	6.11	3.22	2.86	52.7	46.8	1.13	
								1.12
38	bp	7.17	3.74	3.30	52.2	46.0	1.13	1.13
39	dl	7.57	4.00	3.51	52.8	46.4	1.14	
40	dl	7.52	3.97	3.53	52.8	46.9	1.12	
								1.13
41	fk	8.72	4.60	4.11	52.8	47.1	1.12	
42	fk	8.56	4.54	4.00	53.0	46.7	1.14	
								1.13
43	ba	8.22	4.34	3.86	52.8	47.0	1.12	
44	ba	8.31	4.44	3.82	53.4	46.0	1.16	
								1.14
45	be	7.67	4.11	3.53	53.6	46.0	1.16	
46	be	7.74	4.05	3.60	52.3	46.5	1.12	
								1.14
47	cw	(13.61	9.51	4.11	69.9	30.1	2.32)*†	
48	cw	7.45	3.87	3.43	51.9	46.0	1.13	
49	cw	7.02	3.69	3.29	52.6	46.9	1.12	
50	cw	7.21	3.76	3.36	52.2	46.6	1.12	
51	cw	7.73	3.99	3.55	51.6	46.0	1.12	
52	cw	7.83	4.16	3.64	53.2	46.5	1.20	
								1.14

* Not averaged.

† See p. 431.

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
53	ew	9.67	5.21	4.39	53.9	45.4	1.19	
54	ew	8.29	4.27	3.95	51.5	47.6	1.08	
								1.14
55	al	8.97	4.63	4.36	52.0	48.9	1.06	
56	al	9.62	5.22	4.37	54.3	45.4	1.19	
57	al	7.56	4.08	3.47	54.0	45.9	1.18	
								1.14
58	dh	6.78	3.60	3.14	53.1	46.3	1.15	
59	dh	7.69	4.08	3.55	53.1	46.2	1.15	
								1.15
60	dr	4.63	2.48	2.15	53.6	46.4	1.15	1.15
61	du	7.35	3.83	3.34	52.1	45.4	1.15	1.15
62	ea	7.97	4.25	3.68	53.3	46.2	1.15	1.15
63	ec	8.20	4.36	3.78	53.2	46.1	1.15	1.15
64	bd	8.13	4.33	3.79	53.3	46.6	1.14	
65	bd	7.45	3.99	3.41	53.6	45.8	1.17	
66	bd	6.99	3.76	3.19	53.8	45.6	1.18	
								1.16
67	fo	8.78	4.66	4.04	53.1	46.0	1.15	
68	fo	8.96	4.81	4.10	53.7	45.8	1.17	
								1.16
69	fj	8.84	4.74	4.04	53.6	45.7	1.17	1.17
70	fj	(4.51	2.43	1.93	53.9	42.8	1.26)*	
71	aw	7.71	4.15	3.54	53.8	45.9	1.17	1.17
72	aw	7.29	3.92	3.33	53.6	45.7	1.17	
73	db	8.18	4.39	3.78	53.7	46.2	1.16	
74	db	7.63	4.09	3.49	53.6	45.7	1.17	
								1.17
75	di	8.35	4.51	3.78	54.0	45.3	1.19	
76	di	7.54	4.05	3.54	53.7	46.9	1.14	
								1.17
77	dk	8.29	4.52	3.80	54.0	45.3	1.19	
78	dk	8.27	4.41	3.87	53.3	46.8	1.14	
								1.17
79	df	7.26	3.97	3.30	54.7	45.5	1.20	
80	df	7.56	4.03	3.47	53.3	45.9	1.16	
								1.18
81	ee	8.16	4.39	3.71	53.8	45.5	1.18	1.18
82	er	8.54	4.61	3.90	54.0	45.7	1.18	1.18
83	bc	8.02	4.43	3.56	55.3	44.4	1.24	
84	bc	2.09	1.10	0.96	53.1	45.9	1.15	
85	bc	7.83	4.22	3.58	53.9	45.7	1.18	
								1.19
86	cx	9.35	5.07	4.26	54.2	45.6	1.19	1.19
87	dv	(7.68	4.10	3.58?	53.4	46.6	1.15)*	
88	dv	7.66	4.17	3.44	54.4	44.9	1.21	
89	dv	7.44	3.98	3.41	53.5	45.8	1.17	
								1.19
90	ak	8.67	4.67	3.90	53.9	45.0	1.20	1.20
91	dc	6.87	3.74	3.12	54.4	45.4	1.20	1.20
92	dg	8.04	4.39	3.63	54.6	45.1	1.21	
93	dg	7.66	4.15	3.48	54.2	45.5	1.19	
								1.20
94	ef	7.82	4.26	3.55	54.5	45.4	1.20	1.20
95	ay	8.06	4.40	3.64	54.6	45.2	1.21	--
96	ay	8.46	4.60	3.81	54.4	45.0	1.21	
								1.21
97	do	7.95	4.33	3.58	54.5	45.0	1.21	1.21
98	da	8.71	4.79	3.94	55.0	45.2	1.22	1.22
99	dd	7.80	4.26	3.50	54.6	44.9	1.22	1.22
100	ej	8.09	4.45	3.61	55.0	44.6	1.23	1.23
101	es	8.61	4.75	3.86	55.2	44.8	1.23	1.23
102	eu	8.16	4.46	3.63	54.7	44.5	1.23	1.23
103	bz	9.60	4.71	3.81	49.1	39.7	1.24	1.24
104	dq	6.49	3.58	2.88	55.2	44.4	1.24	1.24
105	ed	9.20	5.10	4.13	55.4	44.9	1.23	
106	ed	9.11	5.00	4.01	54.9	44.0	1.25	
								1.24
107	eh	8.22	4.54	3.62	55.2	44.0	1.25	1.25

* Not averaged.

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
108	u	9.74	5.78	3.93	59.3	40.3	1.47	
109	u	9.35	5.09	4.18	54.4	44.7	1.22	1.35
110	el	(1.62	1.00	0.61	61.7	37.6	1.64)*	
111	el	4.45	2.57	1.88	57.8	42.2	1.36	
112	el	4.44	2.55	1.89	57.4	42.3	1.35	1.36
113	fn	(1.25	0.69	0.53	55.2	42.4	1.30)*	
114	fn	10.57	6.18	4.37	58.5	41.3	1.41	
115	fn	10.49	6.11	4.38	58.0	42.0	1.33	1.37
116	bv	11.13	6.05	3.94	54.4	35.4	1.54	1.54
117	bv	No growth						
118	cz	14.93	9.03	6.91	60.4	39.6	1.53	
119	cz	12.54	7.59	4.95	60.5	39.5	1.53	
120	cz	12.28	7.60	4.68	61.8	38.2	1.62	1.56
121	ck	12.14	7.60	4.53	62.6	37.3	1.68	1.68
122	cl	12.55	7.84	4.68	62.5	37.3	1.68	1.68
123	m	10.41	6.59	3.77	63.3	36.2	1.75	
124	m	11.03	6.87	4.14	62.3	37.5	1.66	1.71
125	s	12.14	7.73	4.37	63.7	36.0	1.77	
126	s	10.97	6.87	4.13	62.6	37.6	1.66	1.72
127	dp	13.23	8.49	4.79	64.2	36.2	1.77	
128	dp	12.45	7.91	4.69	63.5	37.7	1.69	1.73
129	o	10.38	6.18	4.13	59.5	39.8	1.50	
130	o	10.45	6.94	3.51	66.4	33.6	1.98	1.74
131	fl	11.91	7.42	4.42	62.3	37.1	1.68	
132	fl	12.12	7.83	4.35	64.1	35.6	1.80	1.74
133	cj	13.20	8.41	4.79	63.7	36.3	1.76	1.76
134	cj	(8.24					)*	
135	c	(7.19	4.95	2.20	68.8	30.6	2.25)*	
136	c	12.26	7.84	4.36	64.0	35.6	1.80	1.80
137	c	4.86	2.86	2.00	58.8	41.2	1.43)*	
138	cs	17.62	11.39	6.26	64.6	35.5	1.82	
139	cs	16.03	10.34	5.69	64.5	35.5	1.82	1.82
140	cu	15.01	9.66	5.28	64.4	35.2	1.83	1.83
141	cy	17.39	11.46	5.90	65.9	33.9	1.94	
142	cy	(0.05					)*	
143	cy	15.31	9.67	5.59	63.2	36.5	1.73	1.84
144	b	11.75	7.50	4.10	63.9	34.9	1.83	
145	b	13.40	8.73	4.63	65.1	34.6	1.88	1.86
146	en	15.85	10.33	5.49	65.2	34.6	1.88	1.88
147	ao	14.78	9.22	5.47	62.4	37.0	1.69	
148	ao	14.18	8.78	5.34	61.9	37.7	1.64	
149	ao	14.79	9.14	5.61	61.8	37.9	1.64	
150	ao	16.46	11.95	4.45	72.6	27.0	2.69	
151	ao	16.96	9.22	4.72	54.4	27.8	1.95	1.92
151	de	14.69	9.64	5.01	65.6	34.1	1.92	1.92
153	bw	13.66	8.96	4.64	65.6	34.0	1.93	1.93
154	n	12.19	8.10	4.10	66.4	33.6	1.98	
155	n	10.62	6.66	3.95	62.7	37.2	1.69	
156	n	13.94	9.48	4.41	68.0	31.6	2.15	1.94
157	bq	14.40	9.53	4.90	66.2	34.0	1.94	1.94
158	cq	17.66	11.70	5.96	66.3	33.8	1.96	
159	cq	14.41	9.50	4.89	65.9	33.9	1.94	1.95

* Not averaged.

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
160	cr	17.68	11.51	6.16	65.1	34.8	1.87	
161	cr	14.55	9.84	4.69	67.6	32.2	2.10	
								1.99
162	bs	15.63	10.41	5.17	66.6	33.1	2.01	2.01
163	bu	14.60	9.76	4.85	66.9	33.2	2.01	2.01
164	cg	15.31	10.21	5.08	66.7	33.2	2.01	2.01
165	ct	14.94	9.99	4.96	66.9	33.2	2.01	2.01
166	bj	No growth						
167	bj	(2.56	2.21	0.31	86.3	12.1	7.13)*	
168	bj	13.44	9.05	4.38	67.3	32.6	2.07	2.04
169	bj	12.50	8.40	4.17	67.2	33.1	2.01	2.06
								2.11
170	dm	14.56	9.79	4.75	67.2	32.6	2.06	
171	by	14.95	10.05	4.77	67.2	31.9	2.11	
172	v	13.11	8.84	3.93	67.4	30.0	2.25	
173	v	12.90	8.62	4.16	66.8	32.3	2.07	2.11
174	v	12.19	8.12	4.04	66.6	33.1	2.01	
175	bl	14.43	9.81	4.61	68.0	31.9	2.13	2.13
176	br	14.26	9.73	4.52	68.2	31.7	2.15	2.15
177	cc	16.12	10.96	5.02	68.0	31.1	2.18	2.18
178	fd	15.55	10.70	4.89	68.8	31.5	2.19	2.19
179	bk	13.44	9.21	4.17	68.6	31.0	2.21	2.21
180	fc	15.20	10.47	4.73	68.9	31.1	2.21	2.21
181	dw	13.40	9.16	4.11	68.4	30.7	2.23	2.23
182	fe	15.78	10.93	4.89	69.3	31.0	2.24	2.24
183	cn	15.22	10.94	4.28	71.9	28.6	2.25	2.25
184	ey	14.69	10.15	4.51	69.1	30.7	2.25	2.25
185	cb	14.90	10.08	4.45	67.6	29.9	2.26	2.26
186	cm	15.51	10.73	4.74	69.2	30.6	2.27	2.27
187	ch	15.09	10.51	4.55	69.7	30.1	2.31	2.31
188	dz	15.22	10.59	4.57	69.6	30.0	2.32	2.32
189	dj	15.30	10.67	4.64	69.7	30.3	2.30	
190	dj	15.30	10.75	4.55	70.3	29.7	2.36	
								2.33
191	aj	20.48	14.06	5.97	68.7	29.1	2.36	
192	aj	19.51	13.47	5.96	69.0	30.1	2.26	
193	aj	21.08	14.60	6.23	69.3	29.6	2.34	
194	aj	19.38	13.25	6.04	68.4	31.2	2.19	
195	aj	23.88	17.85	5.87	74.7	24.6	3.04	
196	aj	14.94	9.76	5.11	65.3	34.2	1.91	
								2.35
197	a	14.55	10.42	4.10	71.6	28.2	2.54	
198	a	19.02	13.53	5.32	71.1	28.0	2.54	
199	a	13.15	9.19	3.88	69.9	29.5	2.37	
200	a	12.06	8.24	3.85	68.3	31.9	2.14	
								2.40
201	et	15.30	10.79	4.50	70.5	29.4	2.40	2.40
202	bo	14.28	10.09	4.15	70.7	29.1	2.43	2.43
203	cv	12.91	9.11	3.83	70.6	29.7	2.38	
204	cv	13.68	9.76	3.94	71.3	28.8	2.48	
								2.43
205	dn	14.49	10.15	4.28	70.0	29.5	2.37	
206	dn	14.01	9.97	4.00	71.2	28.5	2.49	
								2.43
207	ca	14.18	10.06	4.13	70.9	29.1	2.44	2.44
208	ap	18.92	13.36	5.25	70.6	27.7	2.54	
	ap	18.03	12.76	4.77	70.8	26.5	2.67	
								2.61
209	ag	(16.21					)*	
210	ag	17.94	13.12	4.93	73.1	27.5	2.66	2.66
211	w	22.41	15.90	6.54	71.0	29.2	2.43	
212	w	13.62	9.52	3.14	69.9	23.1	3.03	
213	w	21.61	15.80	5.52	73.1	25.5	2.86	
214	w	13.06	9.29	3.83	71.1	29.3	2.43	
								2.69
215	ff	18.66	13.64	4.91	73.1	26.3	2.78	
216	ff	21.35	15.34	5.79	71.9	27.1	2.65	
								2.72

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
217	ci	12.25	9.02	3.20	73.7	26.1	2.82	2.82
218	ce	(1.07					)*	
219	ce	2.32	1.89	0.04	81.5	17.2	4.12	
220	ce	3.54	2.75	0.76	77.7	21.5	3.62	3.87
221	fb	2.28	2.21	0.00	97 0	00.0		
222	fb	4.02	4.01	0.00	99 9	00.0		
223	fb	1.39	1.39	0.00	100.0	00.0		

* Not averaged. † See p. 431.

carbon dioxid, and the volumes of hydrogen all reduced to 0° C. and 760 mm. The percentages of carbon dioxid, and of hydrogen in the next two columns are followed by the values of the ratios CO_2/H_2.

The determinations have been arranged in Table 9 in the order of the ascending average values of the ratio.

RELATIONSHIP BETWEEN THE GAS-PRODUCING POWERS OF THE DIFFERENT GROUPS.

It has been noticed that with the ascent of the ratios there is a very marked increase in the total volumes of gas, and that this is due almost entirely to an increase in carbon dioxid. In other words, the volume of hydrogen is more or less constant. This is shown strikingly in the plotted values, Fig. 7. In this figure the abscissae represent volumes of carbon dioxid and the ordinates volumes of hydrogen. Each point in the plot represents the carbon dioxid and hydrogen of one of the determinations listed in Table 9. Omitting a consideration of the area of scattered dots nearest the origin of the plot which we have previously explained, it will be seen that there is a very marked tendency toward constancy of hydrogen with but very slight increase in the volume of hydrogen to correspond to a marked increase in carbon dioxid. A plot made with only average values emphasizes this to some extent.

A similar relation between the total volume of gas and the value of carbon dioxid is to be found in the work of MacConkey. A recast of his Table 1, according to ascending quantities of gas is given:

Ratio CO_2/H_2.	1/5	1/4	1/3	1/2	2/3	3/2	2/1
Volume of gas modes at	25%	30%	25% and 40-50%	40% 50 " 60 "	60%	87%	90%

This relation, altho not so distinct, is still evident in MacConkey's Table 2 in which are compared a variety of bacteria from various sources. Smith noted a similar relationship between the gas production of *B. coli* and *B. cloacae* and the same may be discovered in the comparisons of Burri and Düggeli. One must be very careful, however, not to confuse this with the *apparent* increase of carbon dioxid over hydrogen which takes place after the medium in a fermentation tube has become saturated with gas and the carbon dioxid is no longer held back.

The fact that even with accurate methods Groups II and III continue to show slight variation in the relation CO_2/H_2, whatever the cause may be, is sufficient evidence that part, at least, of the carbon dioxid is liberated independently of the hydrogen. Were it not so, some constant relationship between the volumes of each gas would be apparent. That such a relationship does exist in the case of the colon group and that this relationship is indicated by the ratio $CO_2/H_2=\frac{1}{1}$ can be made to appear reasonable. Hoppe-Seyler's observation in 1887 that calcium formate infected with river mud is decomposed into carbon dioxid and hydrogen; Scruel's contention that the gases evolved by *B. coli* are the products of the reaction $HCOOH \rightarrow H_2 + CO_2$; and the more thorough investigations of P. F. Frankland and his collaborators, of Pakes and Jollyman, of Harden, and, recently, of Franzen and his collaborators have made it quite probable, that not only *B. coli*, but a large number of bacteria are capable of decomposing the formic acid which they produce into equal volumes of carbon dioxid and hydrogen. Were this the only reaction involved, it would lead to the conclusion that the ratio CO_2/H_2 should be 1/1 during the whole course of the fermentation. Various observers have maintained that the ratio for the colon bacillus is not constant. Stamm, for instance, argues that the CO_2 and H_2 are evolved separately, for he observed that at the beginning of the fermentation there was more carbon dioxid in the gas collected above the medium than later in the fermentation. The gas remaining in solution, if added to this, would have served only to accentuate this difference. But attention has already been called to the possibility of loss of hydrogen by oxidation. On the other hand, Mendel observed a marked change of

ratio with varying concentrations of sugar. This conclusion would somewhat invalidate the theory that the gases liberated by *B. coli* are the products of the reaction $H_2CO_2 \rightarrow H_2 + CO_2$.

As indicated in a previous paragraph, we have not only shown Mendel's results to be of no value, but have found the ratio to be practically unchanged by addition of sugar. It was also shown to be unchanged by variations in the temperature of incubation.

This constancy which we have observed in the ratio for *B. coli* is at least suggestive of a single chief reaction and a close relationship between the carbon dioxid and hydrogen. It would indeed be rash to push this hypothesis beyond the point where it is useful as a temporary foundation upon which to build the plans of future research. But considering it as such, we may go a step farther. The easy gradations illustrated in the plot, Fig. 7, with which Groups II and III lead off from Group I, and the remarkable coincidence that the hydrogen volumes of all the groups are approximately the same make plausible the assumption that the reaction $HCOOH \rightarrow H_2 + CO_2$ holds good for all, and that superimposed upon this in the fermentation by Groups II and III are other reactions in which is evolved only carbon dioxid.

The justification for this hypothesis may be divided into two parts. It already has been made the guide in a search for those substances which the members of Groups II and III decompose with the liberation of CO_2 alone, in the hope that the use of these substances may prove to be a rational means of differentiation. In the second place, the hypothesis serves in taking the first step toward an explanation of certain variations in gas production which may be attributed to variations in the conditions of growth rather than to instability in the phsyiological powers of the bacteria.

Assuming that the gases produced are the product of a single reaction, the ratio of these gases must remain constant whatever the condition. Such is the tendency exhibited by members of Group I. But if two or more reactions are involved, and these are in any way independent, it is perfectly possible that one or all will be subject to variation to different degrees as the conditions surrounding them vary. In this case, the conditions must be rigidly constant to obtain the same ratio of gases in any two cases. The

conduct of *aj*, and the members of Groups II and III point toward the validity of this explanation of the variations. If we accept this hypothesis we see at once that very slight changes in the conditions might affect the extent to which the one reaction would outrun the other. The surprising point would then be not that the ratios varied but that they were so constant.

We wish in particular to emphasize the remarkable tendency in our series for the hydrogen volume to remain constant. This throws upon the CO_2 the responsibility for the change in ratio. Indeed we could almost as well group the organisms upon the basis of their CO_2 production alone, and *it is the CO_2 which the Smith fermentation tube determined with least accuracy.*

CORRELATION OF RESULTS.

In attempting to arrange a collection of bacteria in any semblance of order the question of methods of correlating the mass of heterogeneous observations is of the greatest importance.

So far as systematic bacteriology is concerned the most complete set of records of the characters of various cultures merely adds to the confusion if we are unable so to arrange and classify them that they aid us in grouping the cultures according to their natural relationships. At the very beginning we are confronted with the problem of how distinctly the bacteria are segregated by nature into any grouping that may be considered as genera, species, and varieties. The earlier systematic bacteriologists, following the botanists, with whom they were closely affiliated, divided the bacteria on an almost entirely morphological basis, a plan which the later bacteriologists have for the most part attempted to follow while recognizing the impracticability of carrying it to its logical conclusion. In the earlier days the description of species and construction of genera were based largely on descriptions of isolated cultures with little regard for the real relationship to each other. More recently, however, there has been a tendency to recognize certain more or less well defined groups possessing many fundamental characters in common and doubtless descended from common ancestors.

These groups probably have no sharp limits. Even the higher and more complex plant and animal species are not separated from one another by a sharp line but merge imperceptibly with their nearest relatives on each side. The bacteria, with their simple structure and rapid reproduction, are much more prone to variation, and it is probable that while defined natural groups exist they are surrounded by atypical forms connecting them with other equally distinct groups. How shall we study our cultures to determine the characters, limits, and subdivisions of these major groups? On which characteristics shall we depend for the separation of one from another? To the latter question no categorical answer can be given since the system must be fitted to the group rather than the group to the system. Characters which may be of fundamental importance in classifying the bacteria of one group may be of no value in another. In any attempt at classification each group must be studied, if the information is not already available, to determine which characters are significant and which are valueless.

Of perhaps even greater importance is the manner in which we use our information to form our groups. We may use it to create a confusion from which there appears no hope of forming groups, natural or artificial; we may form arbitrary groups or we may so arrange it that the groups, genera, species, or whatever we may choose to call them, follow the lines of natural descent and relationship. Obviously the larger groups will have a considerable number of characters in common while the subgroups will be connected by the common possession of characters the number and character of which is still a matter of opinion. The usual procedure is to make the final separation on the basis of the presence or absence of a single character. This is illustrated by the method of classification proposed by the standard card of the American Bacteriologists in which two cultures may be widely separated by a difference in a single character. Thus a culture which through degeneration has lost the power of liquefying gelatin is entirely removed from a culture with which it is identical except that the liquefaction of gelatin is retained and the two cultures, tho closely related, are

thrown into separate groups. While the card is useful as a means of recording the characters of bacterial cultures it can never be successful as a method of classification, because it assumes that the significant characters are identical for all groups of bacteria. Furthermore, the practice of making divisions on a single character, sometimes of questionable stability, can only bring systematic bacteriology to such confusion that it is not surprising that some bacteriologists doubt the existence of real species among the bacteria. The possibilities of any system of classification in which final separations are made on the basis of single characters is well illustrated by the arrangement of the colon-aerogenes group, by Bergey and Deehan, who by using 8 characters made 256 possible varieties of which they found 43.

Another good illustration of the arbitrary method of classification is found in the revision of the colon group by Jackson, adopted by the American Public Health Association. In this classification the group is divided into two subgroups, one fermenting dulcite and containing *B. coli communior* and *B. coli communis*, and one failing to ferment dulcite, including *B. aerogenes* and *B. acidi lactici*. *B. communior* is separated from *B. communis* by the ability to ferment saccharose and *B. aerogenes* from *B. acidi lactici* in the same way. Each of these species is farther divided into a large number of varieties on the basis of mannite and raffinose fermentation, indol production, nitrate reduction, and other minor characters. One variety liquefies gelatin, altho it is specified in the list of common characters distinguishing the group that gelatin is not liquefied.

It may be very convenient to have a classification in which a culture can be definitely placed by means of a few tests but it is doubtful if this can be considered more than an expedient if it is not based on the sound foundation of natural relationship. If these separations are not, as they seem to be, on a purely arbitrary basis but follow natural lines, we should expect that if we arrange our cultures by this system each of the four species would be further distinguished by the distribution of the characters which we have found to be constant and distinctive.

Using as distinguishing characters the gas ratio, the fermentation of adonite, starch, and glycerin, the production of indol, and the reduction of nitrates, we obtain Table 10.

TABLE 10.

CORRELATION IN SPECIES OF THE COLON GROUP.

SPECIES	SIGNIFICANT CHARACTER		NUMBER OF CULTURES	CO_2-H_2 RATIO		ADONITE		STARCH		GLYCERIN		INDOL		NITRATES	
	Dulcite	Saccharose		Above 1.4	Below 1.4	+	−	+	−	+	−	+	−	+	−
Communior	+	+	26	13	13	11	14	12	14	6	20	24	2	25	1
Percentage of total				50.0	50.0	44.0	56.0	46.15	53.85	23.08	76.92	92.31	7.69	96.15	3.85
Communis	+	−	16	0	16	0	16	0	16	10	6	13	3	15	1
Percentage of total					100		100		100	62.50	37.50	81.25	18.75	93.75	6.25
Aerogenes	−	+	40	31	8	24	16	19	21	11	29	27	13	39	1
Percentage of total				79.49	20.51	60.0	40.0	47.50	52.50	27.50	72.50	67.50	32.50	97.50	2.50
Acidi lactici	−	−	25	5	19	6	19	0	25	10	15	19	5	22	3
Percentage of total				20.83	79 17	24.0	76.0		100	40.0	60.0	79.17	20.83	88.0	12.0

Of the 26 cultures of our collection that could be classed as *B. communior* we find that one-half have a gas ratio below 1.4, about one-half ferment adonite and starch, 76 per cent fail to ferment glycerin, and nearly all form indol and reduce nitrates. On the other hand, of the 16 cultures which could be designated as *B. communis* all have a ratio below 1.4, all fail to ferment adonite and starch, while 62 per cent ferment glycerin, and nearly all form indol and reduce nitrates.

Forty cultures were classed as *B. aerogenes*, of which 79 per cent had a gas ratio above 1.4. Nearly all of these reduced nitrates but there was little or no evidence of correlation in any of the other tests. A large part of the 25 cultures classed according to this scheme as *B. acidi lactici* have a gas ratio below 1.4; 76 per cent fail to ferment adonite, none ferment starch, and 40 per cent ferment glycerin. If these reactions indicate relationship *B. acidi lactici* should be grouped with *B. communis* rather than with *B. aerogenes*. On the whole these comparisons tend to show that the scheme of classification adopted by the American Public Health Association is based on arbitrary distinctions and, with the exception of *B. communis*, which by an accident seems to be a natural

group, the species created may still contain a heterogeneous collection of organisms.

THE BIOMETRICAL METHOD.

The close relationship shown by the few cultures classified as *B. communis* gives a good illustration of the method which may be used to establish the groups into which the larger group has been divided by nature. A natural group or species is distinguished not by a single peculiar character but by several which have been developed simultaneously under the stress of changing conditions. The type of this group occurs with great frequency while the variants, those who have lagged behind or forged ahead in the development, are found with less and less frequency as they are more and more removed from the type. The study of a large number of cultures with the proper tabulation of the results to show the frequency of occurrence of various combinations of characters as they indicate relationship is the essence of the biometrical method as applied to bacteriology. No particular set of culture characters is required. On the contrary, it is essential to the success of the method that the characters be selected with special reference to the general nature of the bacteria studied. Those that are useful in one group may be of no value in another. This method has been used, unconsciously, by bacteriologists for many years in the gradual establishment of groups with uncertain boundaries of which the colon group is a good example. It is only when it appears under a name of its own that it is looked upon as an innovation. The objection, which may possibly be made, that the species which this method would create would not be sharply defined is a criticism, not of the method by which the species are described, but of the condition in which they exist. A method which will enable us to describe actual species must, necessarily, leave the limits somewhat uncertain, since in nature no species, and especially no bacteriological species, can have hard-and-fast limits.

A botanist selects without much difficulty a type plant from which he makes a description of the species. He usually has before him a large number of plants from which he can choose, not one stunted by unfavorable soil or abnormally large from the influence

of exceptional circumstances, but one which is evidently a fair representative of all those before him. The bacteriologist, on the other hand, sees only the culture with which he is working. To determine the typical culture he must work over a large number of cultures and arrange them in the order of their frequency. The accumulated results of many years' work has established the type and, in an indefinite way, the limits of the colon-aerogenes group.

It is proposed in this paper to point out the existence within the larger group of well defined subgroups marked by co-ordinated characters and separated from the other sub-groups by distinctive characters. Howe, who studied 630 cultures from the stools of 20 individuals, found that this collection was about equally divided between 2 groups differing principally in the fermentation of saccharose and raffinose. The amount of gas and gas ratio was not used, as he considered this of no value. He also states that there is no correlation between mannite, dulcite, starch and other tests and that indol, ammonia, and the nitrates are of no vlaue.[1]

While Howe's two groups no doubt represent the division which could be made in the colon group on the basis of these two tests, there is nothing in the brief abstract available to show that numerous subgroups do not exist within the two groups formed.

It has already been shown that when accurately determined the CO_2/H_2 ratio brings the cultures together in a few circumscribed areas.

By arranging the cultures to show frequency of occurrence, we obtain Table 11 and Fig. 8.

TABLE 11.

DISTRIBUTION OF CULTURES IN RELATION TO CO_2:H_2 RATIO.

0–1	1–1.1	1.1–1.2	1.2–1.3	1.3–1.4	1.4–1.5	1.5–1.6	1.6–1.7	1.7–1.8	1.8–1.9	1.9–2.0	2.0–2.1	2.1–2.2	2.2–2.3	2.3–2.4	2.4–2.6	2.6–2.7	2.7–2.8	2.8–2.9	2.9–3.0	3.0 Up
1	12	37	11	3	0	2	2	7	5	7	6	6	9	4	5	0	3	1	1	1

This shows that a large number of cultures have in common a gas ratio varying within narrow limits and sharply separated from the gas ratio of the other cultures of this collection. In other

[1] In the abstract published in *Science*, the first subgroup of Group II is identical with the second subgroup of Group I. This is evidently a typographical error.

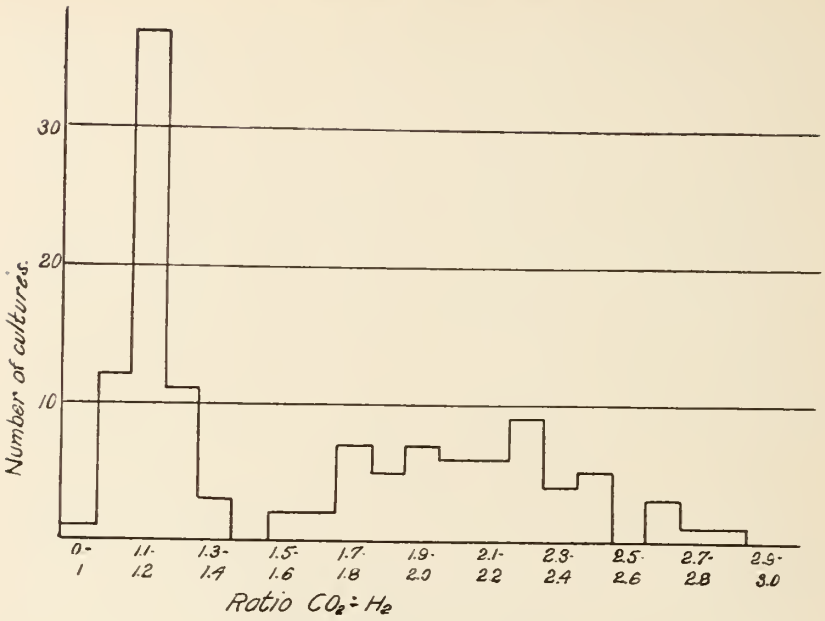


FIG. 8.—Frequency curve for CO_2 : H_2 ratio.

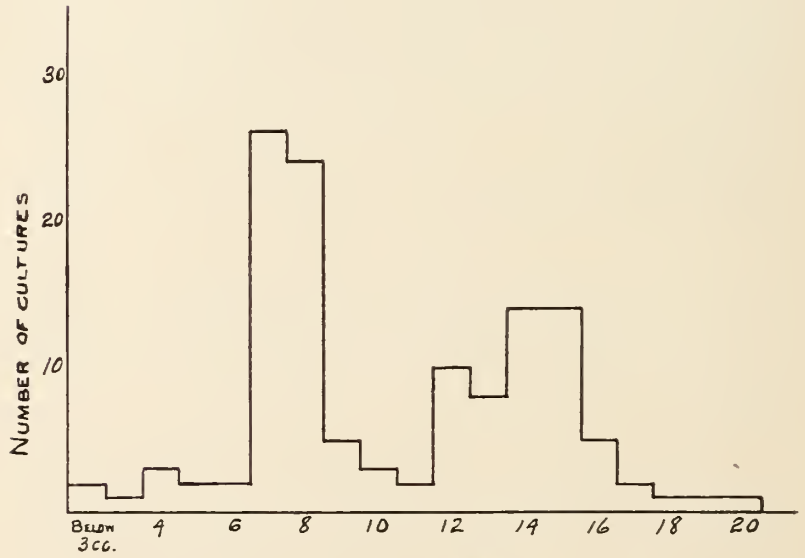


FIG. 9.—Frequency curve for amount of gas.

words, a large number of these cultures bring about an identical or at least very similar reaction when dextrose is fermented. In a similar way we obtain Table 12 and Fig. 9 by arranging the number of cultures forming certain amounts of gas under definite conditions.

TABLE 12.

DISTRIBUTION OF CULTURES IN RELATION TO AMOUNT OF GAS FORMED.

Below 3 c.c.	3 c.c.	4 c.c.	5 c.c.	6 c.c.	7 c.c.	8 c.c.	9 c.c.	10 c.c.	11 c.c.	12 c.c.	13 c.c.	14 c.c.	15 c.c.	16 c.c.	17 c.c.	18 c.c.	19 c.c.	20 c.c.
2	1	3	2	2	26	24	5	3	2	10	8	14	14	5	2	1	1	1

We find here also a sharp mode at 7–8 c.c. of gas and one, perhaps two, between 12 and 18 c.c. By going through the original tables we find, as is shown in Table 13, that of the 65 cultures

TABLE 13.

RELATION OF CO_2 : H_2 RATIO TO AMOUNT OF GAS.

RATIO	NUMBER OF CULTURES	CUBIC CENTIMETERS OF GAS			
		Up to 9.9	10–13.9	14–15.9	16 Up
0.95–1.37.....	65	65 100%	0	0	0
1.5–2.0.....	24	0	15 62.5%	9 37.5%	0
Above 2.....	35	0	6 17.2%	23 65.6%	6 17.2%

which form the mode over 1.1–1.2 in the gas ratio curve, 100 per cent form less than 10 c.c. of gas under the predetermined conditions.

Of the 24 whose gas ratio falls between 1.5 and 2, 15 form from 10 to 13.9 c.c. of gas and nine from 14 to 15.9 c.c. of gas. Thirty-five cultures have a gas ratio above 2 and of these 23, or 65 per cent, form between 14 and 15.9 c.c. of gas, while of the remaining 12 cultures, 6 are slightly above this amount and 6 slightly below. This relation of the gas ratio to the amount of gas formed has already been pointed out and is repeated here merely to show the distinct correlation between these characters.

We have then as the beginning of one subgroup 65 cultures forming less than 10 c.c., almost always 7–8 c.c., of gas with a CO_2 to H_2 ratio of approximately 1.1. If these two characters mark a

natural group we should find that these cultures have in common a number of other characters, either positive or negative, not possessed in common with the other cultures of our collection.

For this purpose we may use to advantage the fermentation of various test substances. Winslow in his work on the coccaceae has used the amount of acid formed to good advantage in separating strains, but with gas formers this is of less value on account of the frequent partial or complete neutralization of the acid in the later part of the incubation. However, we have tabulated the frequency of occurrence for certain arbitrary amounts of acid formed.

Practically all of the cultures ferment dextrose, levulose, galactose, salicin, and lactose. These fermentations are therefore characteristic of the entire group and are of no value in forming subdivisions. Inulin is fermented by so very few that, so far as our cultures are concerned at least, it is of no value. The curve for nearly all of these substances, excepting those in which the mode is obscured by the secondary alkali formation, indicates that the demarkation between fermentation and no fermentation falls at about 0.1 per cent lactic acid. This point has been used in constructing the subsequent tables for all the test substances with the exception of glycerin in which the low point falls at 0.2 per cent. The correctness of this deduction is supported by the fact that no correlation could be found between the fermentation of glycerin and other reactions when the separation was made at 0.1 per cent, while when the division was made at 0.2 per cent certain correlations, which will be pointed out later, became quite evident. If the group of cultures giving a gas ratio of approximately 1.1 is a natural group it should be distinguished from the remaining gas-forming cultures by the possession of several characters not common to the entire collection.

An examination of the tables giving the fermentation of the various test substances shows that the group of cultures with the gas ratio below 1.4 has a distinctly lower fermentative ability than the remaining cultures. This is illustrated by Fig. 10, which shows the relative number of test substances fermented by each group. The average number of the low ratio group, represented by the solid line, ferment 8 or at the most 10 of the 13 substances,

while those with a higher ratio ferment from 9 to 12 of the test substances.

If we consider each of the significant characters, a comparison of the two groups shows that the distinction between them is well defined and specific. This is shown in Table 14, p. 460.

FIG. 10.—Relative number of test substances fermented by different groups.

The difference in the action on nitrates and in the production of indol is not great but shows a slightly higher activity for the low ratio group. With adonite the difference is much in favor of the high ratio group. The high ratio group is more active in the

fermentation of saccharose and raffinose and with starch the difference is very marked. Dulcite and glycerin are fermented with difficulty by most bacteria and we should expect that only a very few cultures of the low ratio group would be able to ferment these two substances. On the contrary, 50 per cent of the low ratio

TABLE 14.

CORRELATION OF CO_2 : H_2 RATIO WITH PHYSIOLOGICAL REACTIONS.

Ratio	Total Cultures	Indol		Nitrates		Adonite		Saccharose		Raffinose		Starch		Dulcite		Glycerin		Gelatin	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Below 1.4	58	51	7	56	2	9	48	23	35	25	33	3	55	29	29	33	25	2	55
Percentage		87.9	12.1	96.5	3.5	15.8	84.2	39.7	60.3	43.1	56.9	5.2	94.8	50.0	50.0	56.9	43.1	3.5	96.5
Above 1.4	56	34	22	51	5	31	25	54	2	51	5	34	22	15	41	10	44	10	42
Percentage		60.7	39.3	91.0	9.0	55.3	44.7	96.4	3.6	91.0	9.0	60.7	39.3	26.8	73.2	18.5	81.5	19.2	80.8

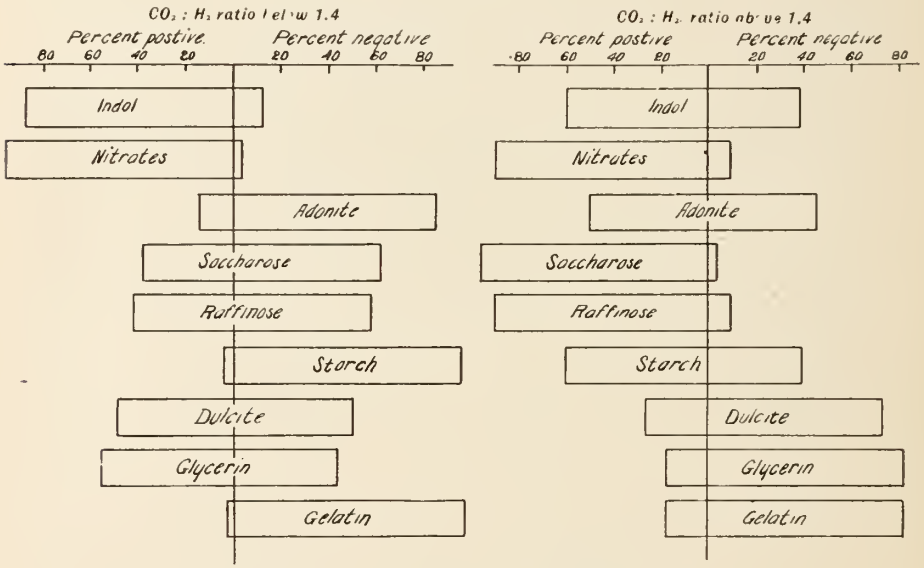


FIG. 11.—Correlation of CO_2 : H_2 ratio with physiological reactions.

cultures ferment dulcite and 57 per cent ferment glycerin, while of the high ratio group 27 per cent ferment dulcite and 18 per cent ferment glycerin. Only 12 cultures liquefy gelatin and of these 10 belong to the high ratio group.

The differences between these groups may be made more clear by reference to Fig. 11 in which the frequency of occurrence of the

significant characters is represented graphically. The number of cultures in each group giving positive reactions with the different tests is plotted to the left of the line, while those with a negative reaction are arranged on the right of the line. This, we believe, shows very clearly that the gas ratio of approximately 1.1 with its correlated characters marks a well defined natural subgroup of the so-called colon-aerogenes group.

It is not improbable that the application of similar methods would result in a more minute division of the group already established. A possible basis for further differentiation may be found in the dulcite fermentation which is positive in one-half of the cultures of the low ratio group. Comparing the fermentative ability of those cultures which ferment dulcite with those that do not, we obtain Table 15.

TABLE 15.

CORRELATION BETWEEN DULCITE FERMENTATION AND OTHER CHARACTERS.

Dulcite	Number of Cultures	Indol		Nitrates		Adonite		Saccharose		Raffinose		Starch		Gylcerin	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
+	29	26	3	28	1	0	28	13	16	14	15	0	29	14	15
Percentage of total		89.66	10.34	96.55	3.45	0	100	44.83	55.17	48.28	51.72	0	100	48.28	51.72
−	29	25	4	28	1	9	20	11	18	11	18	3	26	17	12
Percentage of total		86.20	13.80	96.55	3.45	31.03	68.97	37.93	62.07	37.93	62.07	10.34	89.66	58.62	41.37

In indol production and the reduction of nitrates the two groups do not differ. None of the dulcite fermenters have any action on adonite but they show a somewhat higher activity in fermenting saccharose and raffinose than the cultures giving a negative reaction with dulcite.

Only three cultures of the entire group act on starch and these belong with those failing to ferment dulcite. The dulcite negative group is somewhat more active in fermenting glycerin than the dulcite positive group. While the relation between dulcite and adonite fermentation is striking, the correlation between dulcite fermentation and other characters is hardly strong enough to warrant the assumption of a natural division on results obtained from a comparatively small number of cultures.

TABLE 16.

Correlation of Gas Ratios above 1.4 with Physiological Reactions.

Ratio	Number of Cultures	C.C. Gas from 5 c.c. Broth			Indol		Nitrates		Adonite		Saccharose		Raffinose		Starch		Glycerin		Dulcite		Gelatin	
		10–13.9	14–15.9	16 Up	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
1.5–2	23	14	9	0	17	6	21	2	13	10	21	2	22	1	14	9	5	18	5	18	4	17
Percentage of total		60.87	39.13	0	73.91	26.09	91.30	8.70	56.52	43.48	91.30	8.70	95.65	4.35	60.87	39.13	21.74	78.26	21.74	78.26	19.05	80.95
Above 2	35	6	24	5	21	14	32	3	20	15	32	3	31	4	19	16	4	31	11	24	3	32
Percentage of total		17.14	68.57	14.29	60.00	40.00	91.43	8.57	57.14	42.86	91.43	8.57	88.57	11.43	54.28	45.71	11.43	88.57	31.43	68.57	8.57	91.43

The striking correlations found in the *B. communis* group, as shown in Table 10, are readily understood when one compares them with the distribution of reactions exhibited in Table 14.

Practically all cultures coming under the arbitrary dulcite positive and saccharose negative combination belong in the low ratio group but this method of classification splits up the group based on the gas ratio, which we have every reason to believe a natural one. Any correlations which may occur are accidental and are offset by the lack of correlation in species produced by other combinations of characters.

THE HIGH RATIO GROUP.

The subdivision of the high ratio group presents a much more difficult problem. Natural bacterial groups, like the natural groups of higher organisms, are produced by long existence under uniform conditions; in other words, are closely associated with a definite habitat. Milk is not the habitat of the gas-forming bacteria. On the contrary, the bacteria in milk represent the sum of the contamination of the milk, and consequently include, under the ordinary conditions, a large number of varieties. Our collection of cultures giving ratios above 1.4 doubtless includes representatives of many groups whose limits so overlap that they could not be separated with any certainty without additional study on a larger collection. We have seen also that the gas

ratio, which in the group just considered indicated the principal line of demarkation, becomes more variable as the ratio becomes higher and the reaction more complicated.

Fig. 8 shows some evidences of a separation on the basis of the gas ratio at about 2.0–2.1. The correlation of the two groups made by this somewhat arbitrary division is given in Table 16.

The relation between the ratio and the amount of gas which has already been pointed out holds to some extent with these two groups but beyond this there are no differences which could be considered as distinctive. On the contrary, the percentage of positive reactions for each of the various tests show a surprising similarity especially when they are contrasted with some of the preceding tables in which a distinction between two groups can be made.

Included in the high ratio group are 10 cultures which liquefy gelatin. When these cultures are arranged as in Table 17 so that

TABLE 17.

CORRELATION OF GELATIN LIQUEFACTION WITH FERMENTATIONS.

Cultures with CO_2:H_2 Ratio above 1.4.

Gelatin Liquefied	Number of Cultures	Ratio 1.7–2.2		Adonite		Saccharose		Raffinose		Starch		Glycerin		Dulcite	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
+	10	9	1	2	8	10	0	7	3	6	4	7	3	1	9
Percentage		90	10	20	80	100	0	70	30	60	40	70	30	10	90
−	42	15	27	28	14	39	3	41	1	26	16	3	39	12	30
Percenatge		35.71	64.29	66.66	33.33	92.86	7.14	97.62	2.38	38.09	61.91	7.14	92.86	28.58	71.42

they may be compared with other high ratio cultures which do not liquefy gelatin, we find that they have in common certain characters which tend to separate them from the non-liquefiers. The ratio of 9 of the 10 cultures is identical within narrow limits; nearly all of them fail to ferment adonite and dulcite; they all ferment saccharose and over 60 per cent of them ferment raffinose, starch, and glycerin. The percentage of raffinose fermenters is much higher in the non-liquefiers but the reverse is true in the case of starch and glycerin. A similar relation between the liquefaction of gelatin and the fermentation of glycerin was observed in the streptococci.

CULTURES WITH UNUSUAL GAS PRODUCTION.

In addition to the organism whose gas production has been discussed we have still to describe the gas production of several bacteria which could not be included in Table 9 because they produce no gas in broth containing dextrose. These cultures were isolated from pasteurized milk and are described in a paper by Ayers and Johnson of this laboratory. The conduct of these bacteria when grown in vacuum bulbs shows them to be distinct in many ways from those whose gas production has been described in the preceding pages, and to constitute a very distinct group. The peculiarities which they display cannot be intelligently discussed until further data are obtained. The analyses made will simply be presented for whatever use they may be found to have for purposes of classification and without further comment.

TABLE 18.

THE GAS PRODUCTION OF *fa* IN BROTH.

5 c.c. Standard Broth, 1 Per Cent Dextrose Incubated 7 Days at 30° C. Abundant Growth.

Total Gas	CO_2
c.c.	%
0.32	85.0
0.68	80.0

5 c.c. Standard Broth, 1 Per Cent Lactose Incubated 7 Days at 30° C.

Total Gas	CO_2	H_2
c.c.	%	
1.06	56	Present
1.05	59	"

TABLE 19.

THE GAS PRODUCTION OF *fa* IN 5 C.C. MILK AT 30° C. VACUUM BULB.

Period of Incubation	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
Days	c.c.	c.c.	c.c.	%	%	
7	24.5	14.50	6.66	59.2	27.2	2.18
14	44.07	27.43	16.45	62.2	37.3	1.67
21	28.74	17.18	11.48	59.8	39.9	1.50
31	23.37	13.80	9.56	59.0	40.9	1.44
43	48.36	30.58	17.00	62.9	34.9	1.80
47	45.29	28.25	16.83	62.4	37.2	1.68
47	43.31	27.24	15.93	62.9	36.8	1.71
80	49 28	31.04	18 18	63.0	36.9	1.71

TABLE 20.

GAS PRODUCTION OF *fm*.

5 c.c. Standard Broth, 1 Per Cent Dextrose. No Growth. Duplicates.
5 c.c. Standard Broth, 1 Per Cent Mannite, Incubated 15 Days at 30° C. Vacuum Bulb.

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
21.60.....	10.85	10.61	50.2	49.1	1.02

5 c.c. Milk Incubated 17 Days at 30° C. Vacuum Bulb.

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
42.36.....	25.93	16.43	61.2	38.8	1.58

The slowness with which the gas was produced by this organism when grown in Smith tubes led us to try experiments with large quantities of milk in vacuum with the view of following the course of the reaction from day to day. Accordingly a bulb was prepared holding 200 c.c. of skim milk. After sterilization and inoculation it was evacuated, and then the gas evolved each day was pumped out as completely as possible and analyzed. The enormous amount of gas which was produced each day was more than the pump could manage successfully. Consequently we will omit the analyses, and simply state that at the end of 500 hrs., when the experiment was discontinued, 3,069.9 c.c. of gas had been collected.

A second experiment conducted with 50 c.c. of milk was followed more accurately and this we report below:

TABLE 21.

GAS PRODUCTION BY *fm* FROM 50 C.C. MILK AT 30° C.

Hours	Total Gas to Date	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c.	c.c.	%	%	
6.....	0.14	0.14	.....	.....	.....	.....	.....
30.....	0.25	0.11	.....	.....	.....	.....	.....
48.....	17.80	17.55	10.66	6.87	60.8	39.1	1.55
54.....	35.76	17.96	10.18	7.76	56.7	43.2	1.31
99.....	190.26	154.50	91.16	70.35	59.0	41.0	1.44
121.....	289.57	99.31	59.74	39.57	60.2	39.8	1.51
170.....	484.97	195.40	114.70	85.70	58.7	41.3	1.42
193.....	591.77	106.80	62.82	43.98	58.8	41.2	1.43
217.....	685.87	94.10	53.80	40.30	57.2	42.8	1.33
242.....	772.67	86.80	50.00	36.80	57.6	42.4	1.36
266.....	852.51	79.84	47.90	31.90	59.9	40.1	1.50
289.....	926.62	75.11	44.00	31.00	58.6	41.4	1.42
337.....	1,053.62	127.00	73.40	53.60	57.8	42.2	1.37
362.....	1,141.18	87.56	50.63	36.90	57.8	42.2	1.37
385.....	1,214.80	73.62	42.30	31.30	57.4	42.6	1.35
409.....	1,256.64	41.84	22.70	19.10	54.3	45.7	1.19
433.....	1,266.11	9.47	5.50	3.90	58.9	40.2	1.47
457.....	1,268.60	2.45	1.89	0.60	76.0	24.0	3.16
505.....	1,270.10	1.30	0.95	0.35	72.8	26.7	2.73
553.....	1,271.20	1.30	0.68	0.42	61.8	38.2	1.62
601.....	1,272.45	1.25	0.70	0.55	56.0	44.0	1.27

In Fig. 12, are plotted the total volumes of gas up to the end of each period as ordinates against hours as abscissae.

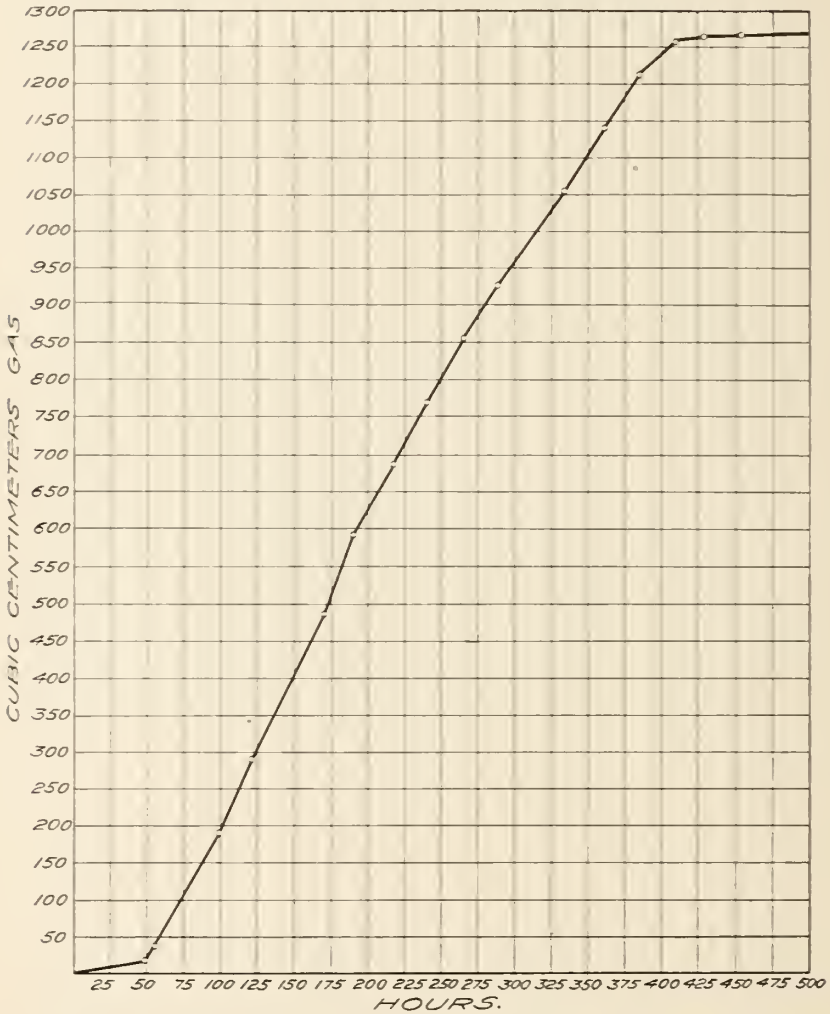


Fig. 12.—Gas production by *fm* from 50 c.c. milk.

CONCLUSIONS.

In drawing conclusions from the data presented, one fact stands out above all others, that certain bacteria when cultivated in dextrose broth at 30° C. furnish a total quantity of gas and a ratio

of the constituent gases which when accurately determined are not found to be remarkably constant. These bacteria find themselves in our Group I with a ratio $\frac{CO_2}{H_2}$ of approximately 1:1.

Other cultures of gas-producing bacteria furnish quantities of gas and ratios of carbon dioxid to hydrogen which, while they are not so constant as those of the first group, are yet clearly distinctive.

Among the cultures furnishing high ratios of $\frac{CO_2}{H_2}$ a few appear distinct because of exceptionally high ratios. Whether these may safely be made a group by themselves on the basis of their gas production alone cannot be said until further data are obtained.

One of the organisms is distinguished by forming carbon dioxid alone and others are distinguished both by qualitative differences in their ability to form gas by fermentation of sugars and by the slow rate of gas evolution.

We have found in the increased constancy and reproducibility of both volumes of gas and ratios of constituent gases, discovered by the substitution of accurate for inaccurate methods of isolation and analysis, a strong suggestion that, just as the older methods furnished discrepant data through their inaccuracies, so the discrepancies of the present data may be traced to the inability of the method to furnish information upon anything but the end products. We have presented a few reasons to support the hypothesis that in those cases where we obtained closely agreeing data we were indeed analyzing the end products of a single reaction while in the more discordant cases we caught only the end products of two or more reactions progressing at different rates. The justification of this assumption is the hope that by further penetration we may be able to discover means of confining the action of the bacteria and obtain but one gas-producing reaction whose end products will appear in the analytical data with the constancy and consequent diagnostic value found in the data for our Group I.

Aside from this the data of the gas determination when given face value appear only to separate our cultures into distinct groups and we have used these as the basis of correlation.

To what extent are we justified in rearranging the group or

establishing new descriptions for species already described? The rule of the botanists and zoölogists of holding to the original description cannot be followed strictly in bacteriology on account of the frequent inaccuracy and insufficiency of the earlier descriptions and even of many of those of later date. Escherich's description of *B. coli communis* was based on culture characters that have been gradually discarded until they are no longer found in the most recent descriptions. So many writers and committees have described this organism, differing one from another, in the characters which are to be considered as significant or as unimportant that the limits of the species, if we may be permitted to call it a species, may be looked upon as a movable function. We find that the ratio of hydrogen to carbon dioxid may vary within wide limits; that *B. coli* always forms indol or that indol formation is unimportant; that it never liquefies gelatin or that it may liquefy gelatin; that it always ferments dulcite or that it occasionally ferments dulcite, and so on. We think, therefore, that we may be permitted to add our version of the salient characters of *B. coli* to the many already published. It should be understood, however, that we make this merely as a suggestion to be used as the basis of further work and not as a final statement of the characters to which *B. coli* must conform. In our opinion the sharply defined group characterized particularly by a gas ratio of approximately 1:1 should be considered, on account of its frequent occurrence and its agreement with the more authentic descriptions, to be *B. coli* in its narrower sense. We may, then, describe *B. coli* as a short, thick rod, gram negative, and usually but not uniformly motile. Indol is produced by nearly all cultures and nitrates are usually reduced to nitrites. Under anaerobic conditions reduction of neutral red almost always takes place. Dextrose is always fermented with the formation of carbon dioxid and hydrogen in nearly equal parts. From 5 c.c. of 1 per cent dextrose broth 6–8 c.c. of gas are produced. Adonite is seldom fermented; lactose, galactose, and levulose are always fermented but many cultures fail to ferment saccharose and raffinose. Starch is rarely fermented but about one-half of the cultures ferment dulcite and glycerin. Gelatin is very rarely liquefied. It grows well on agar and other artificial media. It

is not improbable that two quite distinct varieties exist, one fermenting dulcite and usually failing to ferment adonite and the other failing to ferment dulcite but frequently fermenting adonite. These may be taken to correspond to the old varieties, *communior* and *communis*, but the evidence is not yet sufficient to warrant definite statements.

Closely related to *B. coli* is a group distinguished from it by a marked difference in the gas ratio, which indicates a more complicated reaction, and by a greater fermentative ability in general. This is shown not only in the number of substances fermented and the volume of gas formed but also in the percentage of cultures fermenting the more complex carbohydrates. This difference is especially noticeable in saccharose and raffinose, which are fermented by nearly all cultures, and in adonite and starch, which are fermented by over 50 per cent of the cultures. On the other hand, only a very few ferment dulcite and glycerin. While it is evident that this is a heterogeneous group, we have not yet collected sufficient information to warrant us in making subdivisions. There is some indication of a group formed about the liquefaction of gelatin, but as there were only 10 liquefying high ratio cultures generalizations are unsafe.

It should be noted, however, that these cultures have many characters in common which differentiate them from the remaining high ratio cultures.

While this work has not been sufficient to warrant a complete revision of the colon-aerogenes group, we believe that it marks out the lines along which investigations must proceed if a revision is to be made that will stand the test of time. We need to know, most of all, the mechanism of the reaction which results in the evolution of hydrogen and carbon dioxid, the steps by which the fermentation proceeds, and the products formed in the process. We should know definitely the nature and the source of the by-products causing the alkalin reaction which frequently follows the gaseous fermentation.

The chemistry of the Vogues and Proskauer reaction should be studied until the test can be made accurately and under exact conditions.

When acquired, this information must be so applied that the significant characters can be determined and used to establish a classification that will be lasting because it will separate the group into species made by nature and not by a committee of bacteriologists.

SUMMARY.

A collection of typical gas-forming bacteria occurring in milk was obtained from widely separated and representative sources.

These cultures were examined for morphology, spore formation, Gram-stain, amount of gelatin liquefaction, production of indol, reduction of nitrates and neutral red, and amount of acid produced from dextrose, levulose, galactose, adonite, saccharose, lactose, raffinose, starch, inulin, mannite, glycerin, salicin, and dulcite. Particular attention was given to the production of gas in media containing dextrose, and for the isolation of this gas the exact method of Keyes with some modifications was used.

The carbon dioxid to hydrogen ratio which occurred with the greatest frequency was approximately 1:1. Plotted on the frequency basis this ratio stands apart from all higher ratios.

All cultures giving the 1:1 ratio are distinguished from high ratio cultures by the amount of gas formed under exact conditions. This was uniformly less than the amount produced under identical conditions by the high ratio cultures.

The amount of acid produced from the individual test substances could not be used to advantage because this was frequently obscured by a secondary alkalin fermentation in which the acid was partially or entirely neutralized.

The low ratio cultures fermented a smaller number of test substances than those giving a high ratio, but this difference was not always in those substances usually considered to be fermented with the greater difficulty.

The low ratio cultures are distinguished by a high percentage of cultures giving a positive indol test, reduction of nitrates, and fermentation of dulcite and glycerin. Very few cultures ferment adonite and starch, and only about 40 per cent ferment saccharose and raffinose. The high ratio cultures, on the other hand, give a lower percentage of positive tests with indol, a much higher number

of positive tests with adonite and starch, nearly all ferment saccharose and raffinose but only a very few ferment dulcite and glycerin.

The low ratio group may possibly be divided into those which ferment dulcite but fail to ferment adonite and starch, and those which do not ferment dulcite but occasionally ferment adonite and starch.

No data are available for a logical subdivision of the high ratio group with the possible exception of the small number liquefying gelatin.

The 10 gelatin liquefying cultures agreed very closely in the gas ratio, the fermentation of saccharose and glycerin, and the failure to ferment adonite and dulcite. They differed from other high ratio cultures especially in the gas ratio and the fermentation of glycerin.

The collection contained a few cultures which differed radically from the others in giving a prolonged fermentation in milk with the production of enormous quantities of gas, and one type which was distinguished by the fermentation of dextrose with the production of carbon dioxid only.

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THE DIMINISHED COAGULATION OF THE BLOOD IN ANAPHYLACTIC SHOCK IN THE DOG.*†

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In a recent study carried on in this laboratory an attempt was made to formulate a method of procedure for the investigation of the coagulation of the blood in cases of purpura and allied conditions. Following the publication of that work[1] it occurred to us that it might be of interest to apply the method to a study of the changes in the blood following anaphylactic shock, it being well known that one of the phenomena of anaphylactic shock is the delay or loss of the coagulability of the blood.

This was early (1909) described by Biedl and Kraus,[2] who studied dog's blood, and by Friedberger,[3] who found the same changes in the blood of the guinea-pig. Later studies have fully confirmed these observations and many investigators have advanced explanations. The majority, as for example Arthus,[4] are impressed by the similarity of the changes to those produced by Witte's peptone. Edmunds,[5] who has studied the influence of toxic protein fractions, finds that only in their failure to produce delayed clotting of the blood does the effect of these bodies differ from anaphylactic shock. That it is not an agonal condition was shown by Weiss and Tsuru,[6] who failed to find it in chloroform death. Sirensky[7] found the calcium and magnesium content of the blood unchanged, but the fibrinogen and fibrin ferment slightly decreased. Achard and Aynaud[8] describe an associated disappearance of platelets, but Biedl and Kraus report contrary findings.

Blaizot[9] finds that normal or sensitized rabbits which possess demonstrable fibrin ferment in the blood lose it during anaphylactic shock.

These statements are representative of the views expressed in the literature of this subject and it is therefore evident that the phenomenon is as yet unexplained. Our results do not offer a

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[1] *Arch. Int. Med.*, 1913, 11, p. 305.

[2] *Wien klin. Wchnschr.*, 1909, 11, p. 363.

[3] *Ztschr. f. Immunitätsf.*, I. *Orig.*, 1909–1910, 8, p. 636.

[4] *Presse Medicale*, 1909, 17, p. 305.

[5] *Ztschr. f. Immunitätsf.*, I. *Orig.*, 1913, 17, p. 105.

[6] *Ztschr. f. Immunitätsf.*, 1910, 5, p. 516.

[7] *Ibid.*, 1911, 12, p. 328.

[8] *Compt. rend. Soc. de biol.*, 1909, 67, p. 83.

[9] *Ibid.*, 1911, 71, p. 425.

complete explanation but as they are most suggestive, and throw light on one or two phases of the problem, they are here presented. Our work has shown (1) that the ability of the non-coagulating, post-anaphylactic, oxalated plasma to coagulate can be regularly restored by the addition of small amounts of thromboplastin. The coagulation time of the plasma treated in this way is, however, usually long. (2) Furthermore, we have found that the blood of such dogs as do not develop shock, upon administration of the intoxicating dose, presents certain peculiarities of coagulation.

Our technic was as follows: Dogs were given a sensitizing injection of 5 c.c. of normal horse serum and were then kept under constant conditions for at least 3 weeks. At the end of from 3 to 6 weeks a sufficient amount of blood for study was withdrawn under ether anesthesia from the femoral artery. This was received directly from the cannula into 1 per cent sodium oxalate solution in the proportion of one part of blood to ten of solution. The intoxicating dose of horse serum (5 c.c.) was then injected intravenously and the occurrence of shock determined by observing the fall of blood pressure as recorded by means of a mercury manometer and the usual kymograph. Within 5 min. after the injection, shock being fully developed, blood was again collected in the oxalate solution and the animal killed by chloroform. The specimens thus obtained, one before and the other after shock, were examined as follows: (1) the coagulation time upon the addition of $CaCl_2$ (2 per cent), (2) the influence of the addition of thromboplastin and of fibrinogen was observed, and (3) the content of fibrinogen was measured volumetrically. The coagulation time of the whole blood by means of the Dorrance coagulometer was also determined. The exact technic of these procedures is given in our previous paper.[1]

The results of these studies are given in Table 1 (p. 478).

These figures refer to 7 dogs which showed marked anaphylactic shock. Of 5 other dogs used, 2 gave slight signs of shock, as evidenced by a slight transitory fall in blood pressure; and 3 failed to show any signs of shock.

Of the 7 dogs with marked anaphylactic shock all showed by the Dorrance coagulometer either a delay in, or absence of, coagulability of the blood. The study of the oxalated plasma showed that the addition of calcium and of calcium and fibrinogen was of little benefit in restoring its coagulability. The addition of calcium and thromboplastin solution, however, constantly produced a more rapid coagulation than any other method employed. From this we may assume that the failure of the blood of anaphylactic shock

[1] *Op. cit.*

TABLE I.

Date 1913	Dog No.	Before Intoxicating Dose								After Intoxicating Dose								
		Coagulation Time (in Minutes)								Coagulation Time (in Minutes)								
		Blood	Oxalated Plasma						Fibrinogen Content	Shock	Blood Taken after Injection	Blood	Oxalated Plasma					Fibrinogen Content
			+Calcium		+Ca+Tp.		+Ca+Fibrinogen						+Calcium		+Ca+Tp.		+Ca+Fibrinogen	
			Begin	Complete	Begin	Complete	Begin	Complete			Min.		Begin	Complete	Begin	Complete	Begin	Complete	Percentage
April 7	23	4	2½	3½	1	1½	4½	8	4	Marked	5								6
" 8	24	3½	3	5	2	3	5	9	2	"	3								2
" 14	26	4	7	11	1½	2½	14	42	3	"	1	12	24 hrs.	25	24	58	24 hrs.		3
May 26	45	4½	4	6½	2½	5	5½	30	4	"	4	60	11½ hrs.		5	15	40		6
June 24	60		6	11	3½	10				"	3		21 hrs.	48 hrs.	14	8	24 hrs.		
July 1	63	2½	4	12	4	12				"	2½	6	8½	23	25	28			
	64	5	2½	6½	2	5½				"	2		8	11	4½	10			

to clot is due either to an abnormally small amount of thromboplastin or to an excess of antithrombin. In the former case, according to Howell's theory, the added thromboplastin would have compensated for the existing deficiency; in the latter it would have been sufficient to neutralize the excessive antithrombin and thus allow coagulation. It is obvious, of course, that the addition of calcium and thromboplastin solution did not reduce the coagulation time of the oxalated plasma to that which existed before the administration of the intoxicating dose. This was not to be expected, however, as similar results have been obtained by others investigating the subject of blood coagulation.[1]

As to the second phase of our study, it was found that the blood of the dogs that did not develop shock after the intoxicating dose differed from normal blood in certain important features, coagulability, however, as shown by the oxalated plasma tests, remaining normal. Following exactly the same technic, the oxalated blood was put

[1] Howell, *Am. Jour. Physiol.*, 1911, 29, p. 187; 1912, 31, p. 1; Bayne-Jones, *ibid.*, 1912, 30, p. 1.

into two similar tubes and centrifuged. On removal, one tube presented the usual appearance of a red blood cell sediment with clear, supernatant plasma; in the other, sharply demarcated from the semifluid sediment of red cells, was a solid, cloudy white jelly clot. In a second dog that failed to develop anaphylactic shock, a similar clot was formed in the oxalated plasma of both tubes, indicating a constant association of the two phenomena, which held true also in the third and fourth experiments of this type. In one experiment, in which slight shock was shown by a slight fall in blood pressure, the 1:10 oxalate blood partly clotted. Various control experiments failed to reveal an error in technic capable of explaining this unusual occurrence. It was found, however, that in proportion of 1 part of oxalate solution to 5 parts of blood, coagulation never occurred; whereas in proportion of 1:20 even the normal plasma at times coagulated, tho it never coagulated at 1:10. We therefore felt that if, as is generally believed, oxalate solutions prevent coagulation of the blood by binding the necessary calcium, the failure of certain dogs to develop anaphylactic shock might be shown by these observations to be due to the presence in their blood of an unusual amount of calcium. This would be in accord with reported experiments[1] which seem to indicate that the administration of calcium may prevent anaphylactic shock.

In order to determine this point, quantitative estimations were made of the calcium in blood presenting this peculiar phase of coagulation. As controls, a normal unsensitized animal was studied and several sensitized animals both before and after characteristic shock. The method used was essentially that described by McCrudden,[2] modified in unessential details for adjustment to the small quantity of calcium present in the necessarily small samples of blood available. The results showed no change from the normal, either in the blood of an animal with typical shock or in that of an animal in which the same intoxicating dose had caused no shock and whose blood clotted in the presence of the usual amount of potassium oxalate. In short, no evidence was found to

[1] Kastle, Healy, and Buckner, *Jour. Infect. Dis.*, 1913, 12, p. 127.

[2] *Jour. Biol. Chem.*, 1911–1912, 10, p. 187.

substantiate the hypothesis that there is present in the blood of such animals an excess of calcium.

At the time no other hypothesis could be formulated to explain this phenomenon, but recently MacRae and Schnack[1] have published some observations which may have a distinct bearing upon our results. Briefly, they have found that the addition of calcium-free solutions of a thromboplastic substance, as kephalin, may cause clotting of oxalated peptone plasma, if there is not an excess of oxalate present. The presence of such a thromboplastic substance in the sera which we studied may be a possible explanation of our observation that the plasma of sensitized dogs without anaphylactic shock clotted in 1–10 oxalate solution, but was prevented from clotting by a concentration of 1–5. If this be true it would be of interest to determine whether there is any relationship between the excess of some such thromboplastic substance, as kephalin, and the absence of anaphylactic shock.

SUMMARY.

1. In a series of 12 dogs, 7 developed distinct shock, 2 a slight temporary fall in blood pressure, and 3 showed no evidence of the anaphylactic reaction.

2. Of the 7 dogs developing marked anaphylactic shock, all showed a delay in, or absence of, coagulation of the blood.

3. In all of these the addition of calcium and thromboplastin solution to the oxalated plasma restored its coagulability much more efficiently than calcium alone, or calcium plus fibrinogen solution.

4. No noteworthy change was observed in the fibrinogen content of the blood in this group.

5. Such results point to a decrease of thromboplastin or an excess of antithrombin as the important feature responsible for the loss of power of coagulation in anaphylactic shock.

6. The 5 dogs with slight or no shock showed no changes in the coagulability of the blood, except that in 4 the freshly prepared oxalated plasma (1:10) clotted spontaneously. In no case was

[1] *Am. Jour. Physiol*, 1913, 32, p. 211.

clotting observed in richer proportions of oxalate (1:5). The supposition that this coagulation in the presence of oxalate might be due to an excess of calcium in the blood was shown by the result of quantitative calcium determination to be untenable. The only plausible explanation is that suggested by the work of MacRae and Schnack, that is, that some thromboplastic substance, as kephalin, may be present and in the absence of an excess of oxalate is responsible for the clotting of the plasma.

A NEW METHOD FOR DETERMINING THE RELATIVE STABILITY OF A SEWAGE, EFFLUENT, OR POLLUTED RIVER WATER.*

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(From the Sanitary District of Chicago.)

In my paper entitled, "The Relation of the Nitrates to the Putrescibility of Sewages,"[1] I mentioned briefly the possibility of obtaining accurate information on the biologic-oxygen-consuming power of a sewage by means of the addition of definite amounts of saltpeter. The method has since been tried in the laboratory of the Sanitary District, as well as in field work, with very satisfactory results. The present paper is devoted to the discussion of the technic of the method, the results obtainable, the possibility of its application, and its advantage over other methods.

While comparing the effect upon stability of the dilution of the waters of the Drainage Canal with that produced by adding various amounts of saltpeter representing different amounts of oxygen, I found a very close agreement of the results obtained; i.e., a definite amount of atmospheric dissolved oxygen resulted in an improvement equivalent to the addition of the same amount of saltpeter oxygen as judged by the methylene blue putrescibility test. I assumed at the start that one saltpeter molecule gives off three oxygen atoms in the reduction process. However, on closer and more recent observations I have concluded that only two and one-half atoms are utilized. The amount of methylene blue employed was 0.4 c.c. of a 0.05 per cent aqueous solution per 150 c.c. bottle capacity. My former investigations showed that absolute figures could not be obtained with the methylene blue putrescibility test, because the quantity of methylene blue solution as recommended in the *Standard Methods of Water Analysis* retards decolorization by its germicidal property. When 0.4 c.c. of the coloring matter was added instead of 1.0 c.c., the end point coincided fairly closely

* Received for publication February 21, 1914.

[1] *Jour. Infect. Dis.*, 1913, 13, p. 236.

with the elimination of the total available oxygen in the original liquid. It is clear, therefore, that the relative stability figures assumed to designate the ratio between the available oxygen and the oxygen required for complete oxidation could not be identical where different amounts of methylene blue were employed. They will be lower the smaller the quantity of the dye. Just what the true relative stability figure would be in each individual case can be obtained only by actually determining analytically the amount of available oxygen present at the start and the additional amount of oxygen needed after incubation in a tightly stoppered bottle at 20° C. for 20 days, or at 37° C. for 10 days.

One method, most readily available for determining the biologic oxygen requirements, is the dilution method, which is as follows:

First determine the free oxygen, nitrite, and nitrate oxygen (it is possible that under certain conditions the oxygen available from other sources would have to be taken into account) in the original sewage or effluent. The sum of these constitutes the total available oxygen. Then carefully make different dilutions of the sewage with fresh water, saturated with atmospheric oxygen, in glass cylinders or other suitable vessels. This part of the procedure is tedious and requires great care, as there is danger of artificially aerating the mixture. With a stiff wire, spiral shaped at the bottom, such as I have employed in this method, no appreciable seration occurs in the hands of a careful worker. Nevertheless, the fact remains that the dilutions require time and care and cannot be conveniently carried out in the field. The oxygen content of the fresh water employed for dilution purposes must be noted. After the dilutions have been made, the mixtures are carefully syphoned off into 5-ounce bottles containing 0.4 c.c. of a 0.05 per cent solution of methylene blue. The bottles are properly stoppered, tagged for identification, and shaken to obtain thorough distribution of the dye. We now know the initial available oxygen, as well as the oxygen which was added to the various bottles. After incubation at 20° C. for 20 days, a note is made of the dilution, which was just sufficient to retain the blue color. From this dilution can be calculated the total quantity of oxygen required per liter of sewage or effluent. The ratio of this amount and the oxygen available at the start will constitute the real relative stability. For practical purposes, it is not always necessary to incubate the samples for 20 days, except when the color appears pale during the time of incubation, as the biologic-oxygen-absorption curve is usually flattened out after 10 days to such an extent that the oxygen requirements of the following 10 days appear to be a comparatively small quantity. An experienced observer on routine samples can tell after 10 days' incubation whether or not the color is likely to disappear during the second half of the required incubation period. With a very putrescible sewage or trade-waste it is difficult to prepare high dilutions. A trade-waste may require a dilution of 100 to 500 times its volume of fresh water; in other words, 10–2 c.c. of sewage per 1,000 c.c. of fresh water. Often the character of the trade-waste makes it impossible to obtain a uniform suspension and one can readily see that with such a highly putrescible liquid this might make an appreciable difference in the result. Still

another difficulty is that the wastes of dye-houses, tanneries, gas works, etc., are very often so highly colored that it is impossible to observe the decolorization of the methylene blue. Again, the addition of some wastes or sewages will result in the absorption of the methylene blue by colloids. Provided there is no other coloring matter present, an experienced observer will have no difficulty in telling when the blue color in the sediment has disappeared. Very often, a bluish-green color precedes the formation of the colorless leukobase. Another drawback to this method is that even a skilled worker can hardly make more than four or five such tests during the working day of eight hours. In field work this becomes well-nigh impossible unless the laboratory is close by, altho a sample can be preserved at the start for the nitrite and nitrate examinations. The free oxygen should be determined on the spot, since a delay in this method incurs unreliable results.

Incubations of mixtures of sewage and fresh water for short periods to determine the biologic-oxygen-consumption serve a useful purpose in giving a fair approximation of the "strength" of a sewage, but they do not furnish concrete information on the complete oxygen requirements. Short-time incubation tests are convenient for those eager to obtain quick results, but unless the dilutions, time, and temperature of incubation are uniform, various workers will undoubtedly find it difficult to reconcile results. The rate of exhaustion during the first six hours may not continue during the next six hours or so. Yet there is no doubt that a knowledge of the rate of exhaustion during the first few hours, in connection with a knowledge of the total oxygen requirement of a sewage or stream, serves a valuable purpose. Both should be standardized as methods to furnish a definite basis for adjustment of stream pollution, as attempted by the English Royal Sewage Commission. Such standardization must be undertaken, however, with a view to suiting the conditions peculiar to the United States.

Altho incubations in glass bottles give comparative results, the actual changes in a river will only measure up relatively to the oxygen requirements obtained by such tests. The important factors in the absorption of atmospheric oxygen from a free surface and sunlight are necessarily omitted in the technic proposed by me. These two factors alone may, in many cases, easily account for a difference of 50 per cent in the results between the study under closed and under open conditions. Yet I believe it is better to exclude such variable factors in any attempt to standardize these tests for the sake of obtaining uniformly comparable results.

As previously noted, any method for the determination of the oxygen required for complete oxidation should be simple and convenient. The troublesome features of the dilution method can be largely eliminated by substituting saltpeter oxygen for atmospheric oxygen. A large number of tests, carried on in the laboratory of the Sanitary District and in the field, indicate the superiority of the use of the "saltpeter" method over the "dilution" method.

The technic of the method proposed by me is as follows:

To a number of 150 c.c. bottles containing 0.4 c.c. of a 0.05 per cent solution of methylene blue add varying quantities of saltpeter (a solution of sodium nitrate,

C.P.) representing definite oxygen equivalents. For instance, if the capacity of the bottles used is 150 c.c. and oxygen equivalents are required of 5, 10, 15, 20, and 25 p.p.m., it is best to prepare a stock solution containing 3.19 gm. of sodium nitrate per liter, 1 c.c. of which is equivalent to 1,500 milligrams of oxygen. One cubic centimeter of the solution to 150 c.c. capacity is equivalent to 10 p.p.m. of oxygen. The strength of the sodium nitrate solution can be varied to meet different conditions. When testing the oxygen-consuming capacity of a sewage which may utilize 100 p.p.m. of oxygen or more, it is desirable to employ a nitrate solution of 10 times this strength. If the capacity of the bottle is 150 c.c., the amount of nitrate solution 2 c.c., and the amount of methylene blue solution approximately 1 c.c., there are in the bottle actually only 147 c.c. of sewage, representing the quantity upon which to base calculations. The nitrate solution is fairly stable. Any marked deterioration can be recognized by testing for nitrites which are formed by bacterial reduction. At the time of the collection of a sample of sewage or river water, the nitrites and nitrates should be determined on the spot, or else a sample should be preserved for analysis. The dissolved oxygen is best determined at once. The sample of sewage or river water should be collected in a bottle or cylinder and syphoned off immediately into the various methylene blue bottles, which should be completely filled to prevent settling. The stoppered bottles are shaken to permit an efficient distribution of saltpeter and methylene blue solution, and then placed into the incubator for observation. The more bottles employed with a range of concentration, the better are the chances of accurately determining the biologic-oxygen-consumption or true relative stability. With a badly contaminated river water, it might be wise to add saltpeter in equivalents of 2, 4, 6, 8, 10, and 12 p.p.m. of oxygen, respectively. For a plain domestic sewage, 100, 130, 160, 190, 210, and 240 p.p.m. of oxygen would probably give the desired result. For a trade-waste, much higher dilutions may be required, and it becomes a question of using more bottles until working limits are defined. For colored trade-wastes this method is not suited, as the blue color is obscured. Another method, likewise based upon the principle of nitrate reduction and employed to advantage in such cases, will be described later. Frequently, the rate of oxygen exhaustion, after short-time incubation, may furnish a valuable clue to the approximate quantity of nitrate oxygen required in the "saltpeter" method. Clearly, the procedure of adding saltpeter in place of preparing dilutions with fresh water saves much time, and makes it easily possible to prepare four to five tests in place of one. This, again, means that the average "strength" of a sewage or effluent or stream, which may vary considerably during 24 hours, can be much more closely determined by the "saltpeter" method. The tests can be continued during the night, since even an unskilled man can do the preliminary work, provided the bottles containing the saltpeter solution are prepared in advance.

Table 1 (p. 486) shows the comparative results obtained with both methods on a water highly polluted.

One column was inserted showing the "relative stabilities" obtained when using 0.4 c.c. of 0.05 per cent methylene blue solution. It is clear, of course, that these relative stability figures cannot coincide with the figures in the *Standard Methods*, since in the original tests made by Phelps[1] a stronger concentration of dye was employed. Nevertheless it is of interest to note that the true relative stability as

[1] *Contr. Sanit. Res. Lab. and Sewage Exp. Sta., Mass. Inst. Tech.*, 5, p. 74.

obtained by the "saltpeter" method (average of 40 tests) was 15 per cent lower than the average of the relative stability according to the *Standard Methods*, and by the "dilution" method was 29 per cent lower than the average of the *Standard Methods*.

TABLE 1.

COMPARISON OF "SALTPETER" METHOD TO "DILUTION" METHOD ON POLLUTED RIVER WATER.

DATE, 1913			TOTAL OXYGEN REQUIRED P.P.M.		REL. STABILITY 0.4 C.C. METH. BLUE PER 150 C.C. CAPACITY	INITIAL AVAILABLE OXYGEN P.P.M.
Month	Day	Time	Dilution Method	Saltpeter Method*		
Aug.	4	2:00 P.M.	7.7	5.4	54	3.2
"	5	10:00 A.M.	6.2	4.4	44	2.2
"	5	3:00 P.M.	5.9	4.4	44	2.2
"	6	11:00 A.M.	8.4	6.4	36	2.0
"	7	9:00 "	9.0	6.8	25	1.3
"	11	2:00 P.M.	8.5	7.1	53	2.7
"	12	9:00 A.M.	6.4	4.5	44	2.3
"	13	10:00 "	9.0	6.7	44	1.3
"	15	8:00 "	7.8	5.8	26	1.4
"	20	10:00 "	6.8	5.1	47	1.8
"	20	2:00 P.M.	8.1	6.9	50	2.5
"	22	8:00 A.M.	9.1	6.9	35	1.4
"	25	9:00 "	7.1	5.7	56	3.5
"	25	11:00 "	5.7	5.9	64	4.2
"	25	2:00 P.M.	5.2	4.7	71	3.6
"	26	2:00 "	7.3	5.4	56	2.8
"	27	10:00 A.M.	7.0	5.6	44	1.7
"	27	2:00 P.M.	8.0	7.3	59	2.9
Sept.	4	3:00 "	5.1	5.7	60	3.5
"	5	2:00 "	6.8	5.0	44	2.8
"	7	8:00 A.M.	6.1	3.5	51	0.7
"	7	10:00 "	5.5	3.9	60	1.7
"	7	2:00 P.M.	4.7	4.3	60	2.1
"	8	9:00 A.M.	5.0	4.5	75	3.4
"	9	9:00 "	6.5	4.9	77	2.3
"	10	2:00 P.M.	5.4	5.5	75	3.3
"	11	2:00 "	5.9	5.5	71	3.2
"	12	9:00 A.M.	5.6	5.5	64	2.9
"	12	11:00 "	5.8	6.0	75	3.8
"	12	2:00 P.M.	6.5	5.6	71	3.4
"	13	9:00 A.M.	6.2	5.5	64	3.3
"	13	2:00 P.M.	6.4	5.8	75	3.6
"	15	9:00 A.M.	6.0	5.5	90	4.4
"	16	11:00 "	6.0	6.6	64	3.3
"	16	2:00 P.M.	6.3	6.4	77	4.2
"	17	9:00 A.M.	7.8	5.4	60	2.1
"	17	11:00 "	5.8	5.1	75	2.9
"	17	2:00 P.M.	6.9	5.8	71	3.6
"	18	9:00 A.M.	6.9	5.9	68	3.7
"	18	2:00 P.M.	7.0	6.1	75	3.9
Average			6.7	5.6	59	2.8

* Calculated on basis of N_2 equivalent to O_5 for the nitrates and N_2 equivalent to O_3 for the nitrites.

Average:

Relative stability by Standard Methods 59
Relative stability by "Dilution" Method 42
Relative stability by "Saltpeter" Method 50

With river waters the application of this method is somewhat simpler than with sewages, as they contain comparatively little coarse suspended matter. In a sewage containing an appreciable quantity of dark sediment, the observer, particularly if unskilled, can note more easily the decolorization in "dilution" bottles than

in "saltpeter" bottles. To obviate this difficulty, I worked out a modification of the "saltpeter" method, which is given later.

In Table 2 is shown the relative oxygen consumption obtained by the two methods with the rather dilute domestic sewage of the Thirty-ninth Street sewerage area.

TABLE 2.

Comparison of "Saltpeter" Method and "Dilution" Method on a Weak Domestic Sewage.

Total Oxygen Required by "Dilution" Method P.P.M.	Total Oxygen Required by "Saltpeter" Method* P.P.M.
93	90
91	80
84	87
103	90
116	105
90	89
110	94
109	107
109	100
113	109
91	77
123	104
107	99
85	77
92	85
85	78
127	106
108	105
97	102
156	146
96	84
127	99
134	122
102	101
Average, 106	97

* Calculated on basis of N_2 equivalent to O_5 for nitrates and N_2 equivalent to O_3 for the nitrites.

The general agreement of the "saltpeter" and "dilution" method is somewhat surprising, considering that in making dilutions with fresh water in order to supply the free oxygen, the normal sewage flora is somewhat changed. Particularly in the higher dilutions will the mixture resemble polluted water rather than sewage. In choosing between the two averages, I prefer the "saltpeter" oxygen figure, because it represents the absorption in the original, undiluted sewage.

The fair agreement of the figures seems to indicate that one atom of nitrogen represents 2.5 atoms of oxygen, on which basis I prepared my stock solutions. It still remains to be seen, however, whether the combustion of the organic matter by the saltpeter oxygen will take place in other sewages with like precision, and whether this relation will always hold good. I realize that a biologic reaction, such as this, is likely to be subject to factors not met with in purely chemical reactions. The destruction of saltpeter in sewages has been investigated by various authors; in particular by Letts, Blake, and Totton,[1] who found that nitrogen is evolved, sometimes entirely as free nitrogen, sometimes partly as nitric oxid. Nitrous oxid may also be formed, but the authors' evidence on the point was not conclusive. "The general character of the change is that of a combustion, the oxygen of the nitrate appearing either partly or entirely in the form of carbonic anhydrid." Guth and Keim[2] found it difficult to show experimentally how the individual reactions take place. Their analysis of the gas formed in the saltpeter reduction showed 0.96 = nitrogen and the rest carbon dioxid and oxygen. That there is no ammonia formed during the process of nitrate reduction is amply demonstrated experimentally by Letts, Blake, and Totton,[3] Glaser,[4] Guth and Keim,[5] and Bach,[6] Rideal[7] in his textbook allows 2.5 atoms of oxygen for one atom of nitrogen, and this was also the figure employed by C. B. Hoover in his work at the Columbus sewage testing station. As stated before, I assumed at first that one atom of nitrogen was equivalent to 3 of oxygen, and with the polluted water I obtained, as a matter of fact, a better check with the dilution method by accepting 3 rather than 2.5 atoms of oxygen for each molecule of saltpeter. The work on crude sewages and more detailed comparative tests with two different saltpeter stock solutions convinced me, however, that 2.5 atoms of oxygen for one saltpeter molecule should be allowed.

The question of the inhibiting effect of the larger quantities of saltpeter upon the normal bacterial water and sewage flora would naturally suggest itself. Guth and Keim[8] have shown that even much higher concentrations of saltpeter than employed by me do not diminish the number of bacteria. Saltpeter in such quantities does not act as an antiseptic and the presence of bacteria is essential to the oxidation of the liquid. A few experiments carried on, in which 200 p.p.m. of saltpeter oxygen were added to an artificial emulsion in water, also showed in comparison with a "blank" no inhibiting effect upon the total number of the acid-forming bacteria during the 10 days of incubation at 20° C.

The active gas formation which takes place in a sewage on incubation with saltpeter is plainly visible. Soon the colloidal matter clumps together. The liquid becomes clear, and minute gas bubbles rise continuously to the surface. The continuous ebullition of gas makes the use of seals for the incubation tests highly desirable.

On previous occasions, I have observed, as has been pointed out in this paper at the beginning, that the methylene blue, when employed in quantities of 0.4 c.c. per 150 c.c. bottle capacity, showed no germicidial effect in a sewage. It seemed of interest to test these findings for sewages to which saltpeter had been added artificially. Table 3 shows the biologic-oxygen-consumption in sewage to which 200 p.p.m. of oxygen in the form of saltpeter has been added. The time of incubation was 11 days,

[1] *Chem. News*, 1903, 88, p. 182.
[2] *Gesundh. Ing.*, 1912, 35, p. 52.
[3] *Ibid.*
[4] *Arch. f. Hyg.*, 1913, 80, p. 165.
[5] *Ibid.*
[6] *Gesundh. Ing.*, 1912, 35, p. 341.
[7] *Sewage*, third edition, 1906, p. 131.
[8] *Gesundh. Ing.*, 1912, 35, p. 52.

the temperature 20° C. Methylene blue was added to one of the bottles, and the residual available oxygen determined in this, as well as the "blank" bottles, at the expiration of the incubation.

The small amount of dye added did not interfere with the biologic-oxygen-consumption.

TABLE 3.

BIOLOGIC-OXYGEN-CONSUMPTION OF SEWAGE AFTER THE ADDITION OF 200 PARTS PER MILLION SALTPETER OXYGEN DURING ELEVEN DAYS, WITH AND WITHOUT THE PRESENCE OF METHYLENE BLUE.

Experiment	Bottle	Oxygen Used
No. 1	Blank	92.9
	Blue	94.5
No. 2	Blank	128
	Blue	127

There remain two important questions to be solved: (1) What is the rate of absorption during the period of incubation? (2) Is the biologic-oxygen-consumption independent of the quantity of saltpeter present at the start?

For the purpose of solving the first question, a sewage was distributed into various bottles, a definite quantity of saltpeter added, the mixture incubated at 20° C., and the residual available oxygen determined at certain intervals. I have found on previous occasions[1] that the free oxygen will disappear during the incubation of a sewage independently of the quantity of saltpeter present; therefore, the sum of the residual nitrite and nitrate oxygen at the end of the incubation constituted the total available oxygen.

Table 4 (p. 490) shows the result obtained with crude sewage.

The most important conclusion which can be drawn from a study of this table is that an incubation period of 10 days at 20° C. is practically sufficient to accomplish the oxidation of the unstable matter in sewage. Altho I have made only a few tests to compare the oxygen consumed at 37° C., I judge that a 5-day period may suffice to complete the oxidation at that temperature.

Sewage containing an insufficient amount of saltpeter oxygen showed after incubation for a few days a black "septic" sediment, while the sediment assumed a brown humus-like color if an excess of saltpeter was present. Indeed, the color of the sediment proved such an unfailing indicator of the degree of oxidation that I could tell the approximate quantity of oxygen required, on incubating sewages with varying amounts of saltpeter, without the addition of methylene blue, and by merely selecting the sample the sediment of which did not turn black after 5–6 days incubation. It was apparent that the oxygen requirements of a sewage could be closely determined without any analytical tests, by merely noting the amount of saltpeter oxygen necessary

[1] *Op. cit.*

to prevent the black discoloration of the sediment on incubation. Still closer results could be obtained by determining analytically the residual nitrite-nitrate oxygen in the liquid which remained. The initial free oxygen, nitrate and nitrite oxygen in the average sewage is ordinarily so small that it forms a negligible quantity when compared to the total oxygen requirements.

TABLE 4.

BIOLOGIC OXYGEN REQUIREMENTS OF A SEWAGE ON INCUBATION WITH AN EXCESS OF SALTPETER.

Serial No.	Saltpeter Oxygen Added P.P.M.*	Total Available Oxygen P.P.M.	Days of Incubation	Total Biologic-Oxygen-Consumption P.P.M.	Percentage of Oxygen Consumed
I.	200	204.7	0	.....	.....
			1	74.4	54
			2	95.9	70
			3	98.4	72
			5	100	73
			10	137	100
II.	200	204.0	0	.....	.....
			1	83.9	43
			3	133	68
			5	164	83
			10	197	100
III.	200	204.4	0	.....	.....
			11	133	95
			20	140	100
IV.	200	203.7	0	.....	.....
			11	191	97
			20	196	100
V.	200	203.4	0	.....	.....
			10	149	98
			20	152	100
VI.	200	203.0	0	.....	.....
			10	157	99
			20	158	100
VII.	200	202.2	0	.....	.....
			1	142	.....
			2	150	.....
			3	181	.....
VIII	200	201.4	0	.....	.....
			1	121	.....
			2	171	.....
			3	183	.....
IX.	200	207.7	1	57.8	61
			2	80.2	84
			3	84.2	88
			5	91.7	96
			10	95.1	100
			20	95.1	100

* Calculation on basis of equivalents $N_2=O_5$ for nitrates and $N_2=O_3$ for nitrites.

In order to obtain more definite information concerning the time required for the blackening of the sediment in the bottles on incubation as compared to the decolorization of the methylene blue, a series of close observations were made. These observations are recorded in Table 5.

The results in Table 5 indicate that either the disappearance of the blue color or the darkening of the sediment may serve as a guide. While this undoubtedly will

hold good with most domestic sewages, it should be pointed out here that there may be exceptions. It is always desirable to run a series of comparative tests before adopting this method as a routine procedure. If fairly good checks can be obtained the observation of the darkening of the sediment in the bottles during incubation works out well, and is preferable when the blue color is obscured. As a rule, the sediment will begin to darken around the edge of the bottom. The darkening becomes more apparent when such a bottle is compared to the one containing the next higher concentration of saltpeter, by holding both against the light. The slightest darkening is coincident with the elimination of all of the available oxygen. Whenever the sediment turned black the liquid smelled putrid, thus furnishing an additional index of incomplete oxidation. When in doubt as to which bottle to take for the determination

TABLE 5.

COMPARISON OF TIME REQUIRED FOR APPEARANCE OF BLACK SEDIMENT AND DECOLORIZATION OF METHYLENE BLUE.

SAMPLE COLLECTED	TIME REQUIRED FOR SEDIMENT TO TURN		TIME OF DECOLORIZATION OF METHYLENE BLUE	SALTPETER OXYGEN ADDED IN P.P.M.
	Slightly Black	Noticeably Black		
Crude sewage		5 days	5½ days	75
" "	41 hours	47 hours	37 hours	75
" "		89 "	84 "	125
" "	6½ days		6½ days	125
" "	36 hours		36 hours	75
" "	4 days	4½ days	90 "	125
" "	7 "		7 days	75
" "	4 "		84 hours	75
" "		5½ days	5½ days	75
" "		5½ "	5½ "	175
" "		72 hours	72 hours	125
" "	52 hours		52 "	75
" "	8½ days		8½ days	175
" "	84 hours	5 days	84 hours	175
Settled "		48 hours	48 "	50
" "	36 hours		36 "	50
" "	6½ days		6½ days	50
" "	36 hours		36 hours	50
" "		60 hours	60 "	100
" "	60 hours		60 "	100
" "		60 hours	60 "	50
" "	50 hours	65 "	60 "	50
Septic "	5½ days	6 days	5½ days	50
" "	5½ "	6½ "	6½ "	50

of the residual nitrate oxygen where quantitative determinations are desirable, colorimetric determinations of the nitrites will often serve as a reliable guide. Where the nitrites in one bottle are absent, or present in traces, one can safely take the higher concentration for quantitative determination. In liquids in which the sediment is not turned black in spite of the elimination of the available oxygen, a rough nitrite determination will indicate the concentration to be selected. Sewages containing sufficient saltpeter oxygen had a distinct humus odor at the conclusion of the incubating period.

A method based upon this principle ought to be quite useful in sewage works which lack scientific laboratory control. It is also adaptable for sewages in which the decolorization of methylene blue is difficult to observe. The choice of methods to be employed for the determination of the biologic-oxygen-consumption will also depend

a good deal upon factors such as the iron content of a sewage or the presence of hydrogen sulfide and organic sulfur. A high inorganic iron content is helpful when the septicization of the sediment is to serve as an index of the oxygen requirements, since it is the ferrous sulfide which gives the black color to the sediment.

I have had a slaughter-house waste under observation, the sediment of which would not turn black on incubation, but remained as a grayish-brown floc. As yet I have made no investigation as to why the sediment failed to turn black. I believe that the sediment does not turn "septic" on account of the high sodium chlorid content of the waste, which often reaches 2,000 p.p.m. The sodium chlorid possibly acts as a preservative. I have had very good success with the trade-waste described, when adding methylene blue. The waste is yellowish, turning, after the addition of the dye, to a greenish color. The disappearance of the greenish color on incubation is very distinct.

Again, I have dealt with the effluent of a "biolytic" tank which was entirely free from colloidal matter, containing a little fine black sediment and large quantities of hydrogen sulfide. With this effluent, the total absorption of free oxygen is comparatively slight, since there is little oxidizable matter besides the hydrogen sulfide. The lack of suspended matter in the effluent led me to resort to the "methylene blue" modification of the method described herein. The blue color disappeared immediately in the bottles containing this effluent with various amounts of saltpeter, on account of the presence of hydrogen sulfide. The liquid in the bottles gradually became cloudy, free sulfur being precipitated. The blue color returned usually within an incubation period of 24 hours, to disappear again in the bottles containing the quantities of saltpeter insufficient to oxidize the effluent, altho it remained in the bottles containing the large quantities of saltpeter. The figures obtained with the consumption of oxygen in the "fresh-water" method and the "saltpeter" method compare well.

Having satisfied myself that the quantity of saltpeter oxygen consumed coincides fairly closely with the quantity of fresh-water oxygen, it became necessary to establish the ratio of exhaustion and the ultimate oxygen requirements of a sewage when employing an excess of saltpeter. Table 6 shows the results obtained.

The initial available oxygen, such as recorded in Table 4, has not been determined, as it is practically negligible when compared to the large quantities of saltpeter oxygen added previous to incubation. Considering the difficulties of accurately determining large quantities of residual nitrate in the liquid after incubation as a routine procedure, the figures obtained on adding various quantities of saltpeter to one and the same sewage are consistent. It may, at times, be sufficient to determine the biologic-oxygen-consumption of a sewage by incubating only one bottle with a considerable excess of saltpeter. This method seems attractive by its simplicity. However, I should not recommend it for the present, until more work has proven its reliability. I believe that more accurate figures can be obtained by employing various concentrations of saltpeter

and selecting the one closest to oxygen exhaustion after incubation for 10 days.

TABLE 6.

OXYGEN REQUIREMENTS OF SEWAGE ON INCUBATION WITH AN EXCESS OF SALTPETER.

Serial No.	Source	Total Available Oxygen P.P.M.	Days of Incubation	Total Biologic-Oxygen-Consumption in P.P.M.
I	Crude sewage	200	5	154
		300	5	160
		400	5	158
II	" "	200	5	171
		300	5	180
		400	5	191
III	" "	75	10	68
		125	10	76
IV	" "	125	10	98
		175	10	89
V	" "	125	10	124
		175	10	131
VI	Filtered "	300	9	180
		400	9	189
VII	" "	300	9	181
		400	9	191
VIII	" "	300	9	179
		400	9	203
IX	" "	300	9	180
		400	9	185
X	" "	200	5	90
		300	5	77
		400	5	109
XI	" "	200	5	79
		300	5	75
		400	5	89
XII	" "	200	5	83
		300	5	72
		400	5	80
XIII	Settled "	150	10	144
		200	10	139
XIV	" "	100	10	96
		150	10	109
XV	Septic "	150	10	135
		200	10	139
XVI	Settled "	100	10	82
		150	10	77
XVII	" "	150	10	108
		200	10	118
XVIII	Crude "	175	10	151
		225	10	151
XIX	" "	125	10	124
		175	10	122

We have still to consider the problem of obtaining the relative stability in colored trade-wastes where the decolorization of the

methylene blue cannot be observed. The method which would suggest itself is simple enough, considering the difficulties presented by the physical characteristics of the liquid.

Into a number of bottles containing the trade-waste, add varying quantities of saltpeter. The number of bottles will depend upon the accuracy desired for the determination of oxygen consumption. The same holds true for the various quantities of saltpeter to be added. The smaller the variations in the quantities, the more accurate are the determinations, the greater also is the possibility of missing the required concentration. The bottles are now incubated at 20° C. for 10 days, and the residual nitrate and nitrite in each tested qualitatively. The liquids which on rough quantitative or qualitative tests show the absence of nitrites or only traces are discarded immediately. The bottle giving a marked nitrite reaction is selected for determining the quantity of oxygen required for biologic-oxygen-consumption. Actual quantitative determinations of the nitrate and nitrite will result in a still closer figure.

I believe, however, that with trade-wastes more actual information can be gained by determining the oxygen consumption through many tests, rather than through a few very accurate tests, as wastes vary greatly within short intervals. The physical appearance of the trade-wastes often indicates to the experienced observer whether it is "strong" or "weak." I always prefer to make a few preliminary tests; for instance, with certain wastes the biologic "oxygen consumed" may vary between 400 and 1,000 p.p.m., and in a waste of that kind a figure to the nearest hundred is frequently close enough, whereas, with domestic sewage, the permissible limit may be the nearest 10 p.p.m. Ordinarily, it is not necessary to determine the available oxygen in a trade-waste, unless the relative stability is wanted, as well as the figure indicating the oxygen consumption in p.p.m. The relative stability is calculated in the usual way; assume that the available oxygen at the start (without the added saltpeter oxygen) is 10 p.p.m., and the total quantity of oxygen consumed (including saltpeter oxygen and initial available oxygen), 500 p.p.m., the relative stability would be 2, since 10 is 2 per cent of 500; hence, 98 per cent of oxygen is necessary, in addition, to obtain complete stability.

In conclusion, I wish to emphasize the necessity of controlling the effluent of sewage works by biologic-oxygen-consumption tests rather than by other routine determinations. The purification of sewage is undertaken in many cases to ameliorate or eradicate a live nuisance. A live nuisance from sewage in a fresh-water course is evident mainly in two ways: (1) by the development of putrid odors and (2) by the elimination of fish life. In both cases, the biologic consumption of oxygen is the all-important element. In general, a nuisance will not develop if some oxygen is present. Oxygen is essential to fish life. Of what use, then, are determinations of organic nitrogen, albuminoid ammonia, or solids in solution and suspension? It is common knowledge that with vegetable

matter and animal matter of the same organic nitrogen content the latter will have much greater biologic-oxygen-absorbing power than the former. Dissolved solids or suspended matter may be stable or, on the contrary, highly putrescible. The determination of the volatile suspended matter is, at best, only a rough indication of the putrescibility of the sediment. Of course, the quantity of suspended matter is required as a routine procedure to determine how much floating or settling matter must be removed. To predict definitely the time and extent of a nuisance, chemical determinations in general are of little value.

In studying sewage treatment, I am interested in but two questions: (1) To what extent has the treatment improved the biologic condition of the stream? and (2) What is the amount of suspended matter in the sewage effluent? The latter information is not always necessary. My belief is that the efficiency of a sewage-treatment plant should be judged primarily by the improvement in the biologic-oxygen-consuming power. Some uniform method of determination should be adopted. I am looking forward to the time when the "strength" of a sewage or effluent will be expressed in "milligram per liter biologic-oxygen-consumed," and when the responsibility for stream pollution by various communities in this country will be apportioned mainly on that basis. This process of standardization will be a tremendous task, not to be undertaken by one man alone, but by an organization of experts skilled in the survey of polluted streams.

SUMMARY.

1. Assuming that 5 oxygen atoms of 2 saltpeter molecules and 3 oxygen atoms of 2 sodium nitrite molecules are utilized, saltpeter oxygen added to a sewage or polluted water in bottles, containing methylene blue as an indicator, will improve the stability of the liquid in practically the same ratio as the free oxygen supplied in the "dilution" method.

2. The true relative stability of a sewage or polluted water can be obtained by the addition of varying quantities of saltpeter to bottles containing 0.4 c.c. of a 0.05 per cent aqueous methylene blue solution, and by recording the quantity of saltpeter oxygen in

the bottle which just retains its color. Ten days' incubation at 20° C. appears to be a sufficiently extended period for all practical purposes. Knowing the initial available oxygen and the total biologic "oxygen consumed," the percentage ratio of the two figures represents the true relative stability. This method is recommended for polluted waters and weak sewages. The quantities of oxygen in the form of saltpeter recommended for trial on polluted waters in general are: 2, 4, 6, 8, 10, and 12 p.p.m., respectively. Deviations from these figures may, of course, be necessary to suit the peculiar circumstances of each case. The use of seals on bottles to be incubated is desirable on account of the tendency of decolorized liquids for reaeration.

3. The biologic-oxygen-consuming power of sewages can also be determined without analytical test by the addition of various amounts of saltpeter (it is suggested that these amounts represent 100, 130, 160, 190, 210, and 240 p.p.m. of oxygen) to glass-stoppered bottles containing the sewage, and incubating the same for 5 days at 20° C. The bottles in which the sediment turns "septic" or in which a putrid odor develops should be discarded. The quantity of saltpeter oxygen in the bottle which contains sediment of a brown humus-like character indicates the oxygen required to obtain complete stability. Such a liquid will also possess a humus-like odor. The residual nitrite-nitrate oxygen may be determined in the liquid which just "held out" when greater accuracy is desired. This method is recommended for sewages containing much suspended matter, for sewage works without laboratory facilities, and as an optional method for the "saltpeter" methylene blue method previously described.

4. In a colored trade-waste, it is best to use various quantities of saltpeter (equivalent to 100, 200, 300, etc., p.p.m., up to 1,000 p.p.m. of oxygen), the amount depending upon the judgment, or the outcome of preliminary tests. Add these amounts to glass-stoppered bottles containing the waste. Then determine roughly the residual nitrite after 10 days' incubation at 20° C. Note the bottle, the liquid of which gave a decided nitrite reaction (qualitative or roughly quantitative). The quantity of saltpeter oxygen which this bottle contained at the start will indicate the oxygen-

consuming power of the trade-waste. The residual nitrite-nitrate oxygen may again be determined when greater accuracy is desirable.

5. The saltpeter-oxygen-consumption on incubation at 20° C. is practically complete after 10 days. Experiments with an incubation temperature of 37° C. have not been made, but it is believed that the time of incubation can be cut perhaps one-half without materially influencing the results. Comparative tests are needed, however, to verify this.

6. The method suggested in this paper should be applied to other sewages in order to determine whether saltpeter oxygen and fresh-water oxygen in equivalent quantities result in an equivalent improvement of the stability elsewhere.

7. It is hoped that in the near future a better understanding of the importance of biologic-oxygen-consumption tests will prevail among sewage chemists and engineers. It seems desirable to express the "strength" of a sewage or trade-waste on this basis, rather than on the basis of routine chemical tests in vogue at present.

Finally, I wish to acknowledge the friendly interest of Mr. Langdon Pearse, engineer in charge of sewage disposal investigations of the Sanitary District, in the progress of this work, and the analytical assistance of Messrs. Frank Bachmann and J. T. Meckstroth. I am particularly indebted to Mr. Bachmann for the compilations of the results and for helpful suggestions.

THERAPEUTIC USE OF CERTAIN AZO-DYES IN EXPERIMENTALLY PRODUCED TUBERCULOSIS IN GUINEA-PIGS.*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. VIII.

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This report presents observations made during the last two years in a series of experiments, described in part in an earlier article[1] and continued and extended since the publication of that paper. As is well known, trypan red is the dye first reported by Ehrlich and Shiga in 1904 as having a "Magna sterilans" action in certain trypanosome infections.[2] Numerous investigators have since that time been working with this dye and its companion, trypan blue, and other related dyes, and an extensive literature has developed. Much of this literature, however, was reviewed briefly in my preliminary communication and it has recently been reviewed by Henius[3] and others, so that it seems unnecessary at this time to do more than mention the work which deals with the relation of these dyes to tuberculosis. Goldmann published several monographs on the distribution of the dyes and their use in the study of cells, and in 1912[4] he made an extensive study of bovine and avian tuberculosis in white mice, especially with respect to the development of the disease and the histology and histogenesis of the tubercle. Bowman, Winternitz, and Evans[5] in 1912 and in 1914 made observations with these dyes on the histogenesis of tubercles and tuberculous giant cells. Lewis[6] in

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[1] *Jour. Infect. Dis.*, 1913, 13, p. 378.

[2] *Berl. klin. Wchnschr.*, 1904, 41, pp. 329 and 362.

[3] *Ztschr. f. Chemoth.*, II, *Ref.* 1913, 2, 1037, and 1914, 2, 1097.

[4] *Beitr. z. klin. Chir.*, 1912, 78, p. 1.

[5] *Centralbl. f. Bakteriol.*, 1, *Orig.*, 1912, 65, p. 403, and *Jour. Exp. Med.*, 1914, 19, p. 283.

[6] *Arch. Int. Med.*, 1912, 10, p. 68.

1912 was able to show that trypan red penetrated to the center of fully formed tubercles in rabbits. In 1912[1] also he presented before the American Society for Pharmacology and Experimental Therapeutics a brief report of his early work on modifications of the trypan red moleculi. Compounds of trypan red with iodin, thymus, eucalyptol, guaiacol, and iodoform were prepared and found to have similar powers of penetrating the tubercle. But neither trypan red nor these modifications had any appreciable influence in prolonging the course of the infection. In my earlier paper I showed that both trypan blue and trypan red penetrated tubercles in guinea-pigs, even the soft caseous substance in the centers of tuberculous lymph glands being stained deeply blue or red, and the large necrotic areas so common in the spleen and liver in these animals taking the stain especially well. The tubercles were found well stained a few hours after a single dye injection and retained the stain for many weeks after the last injection. The tubercle bacilli, however, were shown to be but poorly stained by these dyes in the animal body, in the culture tube, or when fixed to a slide; it was also found that the dyes had practically no bactericidal power over these organisms, since guinea-pigs inoculated with bacillary suspensions which had been exposed for 24 hrs. to 1 per cent or even 2 or 3 per cent solutions quickly developed the disease and died with a local and general tuberculosis. The dyes were found lacking even in inhibitory influence, since a luxuriant growth developed on agar tubes which were deeply dyed.

However, as Ehrlich and Shiga had shown that trypan red failed to kill trypanosomes *in vitro* and yet was a very efficient trypanocide *in vivo*, it was thought possible that a similar finding might be obtained in the treatment of tuberculosis. Hence numerous guinea-pigs which had been recently inoculated with human tuberculosis were treated with trypan blue or trypan red; 1 per cent water solutions of the dyes were injected subcutaneously, 3–6 c.c. constituting the dose according to the size of the animal. The injections were repeated once or twice a week and ill effects were rarely observed. Table 1 (p. 500) gives briefly the results.

[1] *Jour. of Pharmacol. and Exper. Therap.*, 1912–13, 4, p. 353.

In all these guinea-pigs the dyes had penetrated the tubercles well. Even the softened caseous substance in the center of the tuberculous lymph glands was deeply stained, while the smaller tubercles had the characteristic structure. At the periphery were cells with coarse, deeply stained granules, differing from the normal granular cells in the size and irregularity of the granules.

TABLE 1.

SHOWING RESULT OF INJECTION OF 4 C.C. OF 1 PER CENT TRYPAN BLUE.

Time between Inoculation and First Dye Injection	Number of Injections	Time between Inoculation and Death	Autopsy Findings	Effect of Dye Treatment
Days		Days		
1	9	35 (killed)	Loc. and gen. tbc.	None
1	9	70	" "	"
1	2	10	No tbc.	Probably caused abortion
1	2	8	No tbc. Pneumonia	None
1	8	72	Loc. and gen. tbc.	"
62	2	72 (killed)	" "	"
62	2	72 "	" "	"
62	2	72 "	" "	"
62	5	104	" "	"
62	2	72 (killed)	" "	"
TRYPAN RED.				
1	10	68	Loc. and gen. tbc.	None
1	10	82	" "	"
1	10	68	" "	"
1	10	82	" "	"
1	6	35 (killed)	" "	"
1	10	75 "	" "	"
1	10	107	" "	"
62	2	72 (killed)	" "	"
62	2	72 "	" "	"
62	2	72 "	" "	"
CONTROLS FOR TRYPAN BLUE AND TRYPAN RED SERIES.				
		63	Loc. and gen. tbc.	
		31	" "	
		82	" "	
		68	" "	
		64	" "	

Within this area was an irregular zone showing almost no stain, but in which fat droplets and cells containing fat could be demonstrated by the use of fat dyes. Then the innermost portion of the tubercle was usually deeply stained, having either a homogeneous or a finely granular appearance. This inner part of tubercles, as well as the large necrotic areas in liver and spleen, had a different and brighter color than the normal portions of the organs. The

giant cells, when present, showed a diffusely stained protoplasm and more deeply stained nuclei. Both in the giant cells and in the central portion of the tubercles, acidfast bacilli could be demonstrated by means of the Ziehl-Nielssen stain. In most of the guinea-pigs in this experiment the dye injections were begun on the day following the infection and were continued at intervals of four or five days for two months. In spite of the early beginning of treatment and the fact that the animals were kept saturated with the dyes for the first two months, all animals developed a local and general tuberculosis, except two which died early of an accidental infection. No influence on the tuberculous process, therefore, could be observed, unless we may regard as a favorable influence the fact that the treated animals lived somewhat longer than the controls. In a disease as irregular and freakish in all its manifestations as tuberculosis, I cannot regard length of life as an indication of curative influence, unless the autopsy findings indicate some definite tendency to healing or arrest of development of the process. In every case in this experiment the autopsy showed an active and advancing tuberculosis. One experiment was made to determine what would be the effect of one maximum dose of trypan blue very early in the disease. Four guinea-pigs were therefore inoculated subcutaneously with human tubercle bacilli and the next day each received an intraperitoneal injection of 10 c.c. of 1 per cent solution of trypan blue. Three of these pigs died during the first week or two from the toxic action of the dye, but the fourth lived 120 days, showing at autopsy a very pronounced local and general tuberculosis.

Through the kind courtesy of Dr. McCurdy I received for trial a bottle of the "vitalrothzenkerfixierbar," which he had used in some of his experiments. Six tuberculous guinea-pigs were treated with 1 per cent solution of this dye in the same way as with the trypan blue. The effect on the normal tissues was very similar to that of trypan blue and trypan red, certain connective tissue cells exhibiting deep red granules such as are not seen after staining fixed tissues. The tubercles, on the other hand, were much less deeply stained than the normal tissues and much less

deeply than with either trypan blue or trypan red. No effect on the tuberculous process was observed.

We must therefore conclude that trypan blue, trypan red, and "vitalrothzenkerfixierbar" have no therapeutic effect on experimentally produced tuberculosis in guinea-pigs.

The distribution of these dyes is so interesting that one can scarcely help theorizing a little on its meaning. The granular stain in certain fixed connective tissue cells, which is the normal response to the stimulus of these dyes, has been the subject of much difference of opinion among workers with these dyes. Goldmann considered these granules as organula or vital granules of the cell, like the plasmosomes of Ehrlich, vitally stained by the dyes which flow in solution in the blood stream. Subsequent workers have gradually worked away from this hypothesis of Goldmann's; Kyes[1] suggests that the action may be phagocytic, since the cells which show the granular stain are the cells most actively engaged in phagocytosis; and McCurdy[2] states definitely that "the presence of granules of a lipoid-insoluble dye in any cell is an indication of phagocytic capacity in such a cell, the granules being formed by phagocytosis of the dyes from the colloidal solution in which they are present in the lymph." Evans,[3] also, in a report before the American Association of Anatomists in 1913 states that the taking up and storing of dye particles by certain cells is identical with the phagocytosis of larger particles by the cell. Phagocytosis seems the most reasonable explanation of the normal granular stain. In tubercles, in all injured, dead, and necrotic tissue on the other hand, we have a different type of staining—a diffuse, homogeneous, structureless stain, generally of a different and less intense color than that present in the granules. Goldmann regarded this as due to the solution of the granules. McCurdy speaks of it as a "passive type of staining in which nucleus and protoplasm are stained diffusely with no architectural details being shown beyond a darker stain in the nucleus." He states that every cell thus stained is permanently injured, and that the stain depends on the mutual affinity of the

[1] Personal communication. Manuscript not yet published.

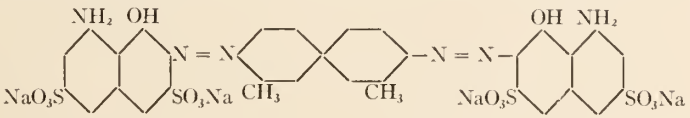
[2] By permission, from unpublished MS. To be published in Nissl-Alzheimer's *Hist. und histopath. Arbeiten über die Grosshirnrinde.*

[3] Abstract of report published in *Anat. Record*, 1914, 8, p. 98.

dye and albuminous degeneration products which may be formed before any morphological change has taken place that is demonstrable by postmortem methods. It has seemed to me possible that a portion of these diffusible colloidal dyes, on entrance into the animal body, change their character to that of a more coarsely colloidal state, the particles of which are readily taken up by phagocytosis, while the rest of the dye circulates in solution in the blood serum and in the lymph and readily permeates injured and dead tissues. It is well known that however thoroughly saturated the tissues become, the blood never contains more than a minimal quantity, merely sufficient to show a faintly blue ring at the periphery of a drop of blood placed on a sheet of white filter paper. The color of this ring is suggestively similar to that seen in injured and dead tissue.

Since trypan blue and trypan red so effectively penetrate the tubercle and yet are so ineffective in treatment of the disease, it occurred to me that their bactericidal and therapeutic power might perhaps be increased by substituting for the sodium sulfonate radicals the sulfonate of other and more bactericidal metals.

For this purpose, silver, copper, mercury, and iron were selected, and Dr. Walter Fraenkel made the desired substitutions for me according to the following methods: The free dye acid was formed from trypan blue dye by covering 5 gm. of the salt with 100 c.c. of fuming HCl, stirring well and allowing it to stand overnight. After dilution with 25 c.c. of water, it was filtered; the precipitate was washed first with 25 per cent HCl and then with equal parts of alcohol and ether. After drying, the dye acid appears in the form of shiny bronze needles, which, unlike the salt, are soluble in methyl alcohol and slightly in acetone and in equal parts of alcohol and ether. The formula of trypan blue is:



Copper trypan blue.—Copper hydroxid ($Cu(OH)_2$) is freshly prepared by adding NaOH to a solution of $CuSO_4$, being careful not to precipitate all the copper. The precipitate is then washed several times with water and then mixed with 3 gm. of the free dye acid and evaporated over the water bath to complete dryness. The residue is extracted in a Soxhlet apparatus with methyl alcohol to remove any unused free dye acid, and the small amounts of $CuSo_4$ and $CuCl_2$ which may be present. After drying, the residue is dissolved in the cold by shaking with about 100 c.c. of water, and filtered, and the filtrate dried in Erlenmeyer flasks by passage of dried air at a temperature of 40°–60° C. The amount of copper present is 12.7 to 12.8 per cent and the formula

is the same as that of trypan blue except that Cu takes the place of Na in each sulfonate group. It consists of bright bronze crystals, easily soluble in water.

Silver trypan blue.—Freshly precipitated silver oxid is carefully freed from NaOH and added to each of two 100 c.c. flasks containing 1.5 gm. of the free dye acid and about 60 c.c. of water. The two flasks are shaken for 5 hrs. in a shaking apparatus. The contents are then filtered and dried at a temperature not exceeding 50° C. The residue is extracted with methyl alcohol and then with acetone. The silver trypan blue thus obtained is a glistening bronze powder having 33.3 per cent silver. The formula is the same as that of trypan blue, except that silver takes the place of the sodium.

Iron trypan blue.—Three grams of free dye acid are mixed with iron oxid and 250 c.c. of water and heated with frequent stirring to dryness. The rest of the procedure is the same as used in making the copper salt and the result is a bright bronze powder containing 7.6–7.9 per cent of iron, and having iron in the trypan blue formula in place of the sodium.

Mercury trypan blue.—Two mercury salts of trypan blue were made, a mercurous and a mercuric. The mercurous salt was made by dissolving 3 gm. of trypan blue in 100 c.c. of water and adding an excess of mercurous nitrate. A finely divided bright blue precipitate results, which is very difficult to filter. The precipitate is washed on the filter with HNO_3 and then with water, and dried in the desiccator. The mercurous salt is insoluble in water and organic solvents and breaks down in alcohol, ether, and other organic compounds. When dried, it forms a bright blue powder containing 48 per cent of mercury. The mercuric salt of trypan blue is prepared in the same way as the mercurous salt except that mercuric nitrate is used instead of mercurous nitrate. The precipitate, after filtering and drying, is bronze colored and insoluble in water and all organic solvents. It can be made also by shaking mercuric oxid with the free dye acid for 3–4 hours. After filtering, it is washed with 1 per cent HNO_3 until excess of mercuric oxid is removed, then with water, and extracted in the Soxhlet apparatus with methyl alcohol and then with acetone. This salt contains 31.6–31.7 per cent mercury. Since both of the mercury salts are insoluble in water, it was necessary to use them in a fine suspension. This suspension was so fine that there was no appreciable settling out of the dye in weeks.

The bactericidal action of these salts of trypan blue was first tested by mixing 0.1 c.c. of a dilute filtered suspension of human tubercle bacilli with 5 c.c. of dye solution, and allowing the mixture to stand for 24 hrs. at incubator temperature. One drop of the mixed dye and bacterial suspension was then diluted with 5 c.c. of sterile normal salt solution and injected subcutaneously into guinea-pigs. The results are stated briefly in Table 2 (p. 505).

The two blanks in the mercury salts are due to the fact that by a mistake, these pigs received a later injection of tubercle bacilli which had been exposed to $CuCl_2$, and a local tubercle developed at the site of this later injection, followed by a generalized infection, causing the death of the animals. Since no tubercle developed at the site of the first injection, it may be assumed that the infection to which they succumbed was due to the second injection and

that the mercury salts have considerable bactericidal or at least inhibitory power over the human tubercle bacilli. This, of course, might readily be expected, since we have here a .3 per 1,000 concentration of mercury in the mercuric salt and a .7 per 1,000 in the mercurous salt, while in a 1 per 1,000 solution of $HgCl_2$ the concentration of mercury is only about 0.7 per 1,000. None of the other salts of trypan blue used exhibited any marked bactericidal

TABLE 2.

Dye Salt	Concentration Used	Time before Local Nodule	Subsequent Treatment	Time before Death	Autopsy Findings
	Per Cent	Days		Days	
Copper trypan blue	1	18	Lithium carmine	115	Sev. loc. and gen. tbc.
	1	11	Brilliant cresyl blue	130	Sev. loc. and slight gen. tbc.
	1	11	Brilliant cresyl blue	112	Sev. loc. and gen. tbc.
Silver trypan blue	1	18	Brilliant cresyl blue	121	Slight loc. and slight gen. tbc.
	1	46	Methylene blue	177	Sev. loc. and gen. tbc.
	1	None	Brilliant cresyl blue	121	No loc., sev. gen. tbc.
Iron trypan blue	1	40	None	156	Sev. loc. and gen. tbc.
	1	26	"	125	" " "
	1	40	"	109	" " "
Mercurous trypan blue	Hg 0.7	None	"	120	No loc., only pulmonary tbc.
	"	"	"		
	"	"	"	240 (killed)	No tbc.; condition excellent
Mercuric trypan blue	0.3	"	"		
	"	"	"	264 (killed)	No tbc.; condition excellent
	"	"	"	240 (killed)	No tbc.; condition excellent
Controls NaCl	Normal 0.9	26	"	73	Loc. and gen. tbc.
	"	26	Pyrrhol blue	137	" "
	"	26	None	93	Slight loc., sev. gen. tbc.
	"	19	"	112	Sev. loc. and gen. tbc.
	"	19	"	118	Slight loc., sev. gen. tbc.

influence over the tubercle bacilli, altho the local tubercle developed rather slowly in some of the animals and in one of the pigs infected with the silver trypan blue treated bacilli, no local tubercle developed.

Experiments to determine the penetrating power of metallic salts of the dye showed that the silver and iron salts penetrated the tubercles as easily and more specifically than does the trypan blue itself, since the normal tissues were usually quite unstained by these salts while the tubercles and necrotic areas were nearly

always deeply colored. The copper salt and the two mercury salts apparently do not penetrate the tubercles at all, nor indeed do they stain the normal tissues. This may be due to the fact that the high local toxicity of these salts made it necessary to use much smaller doses and much more careful administration than any of the other salts required. It may be, however, and more probably is, due to a local breaking down of these dyes into more or less insoluble and difficultly diffusible compounds, which remain at the point of injection. This may also explain the extensive necrosis and sloughing of the skin so common at the point of injection of these salts, and very rarely occurring with trypan blue or the silver and iron salts.

To determine whether these metallic salts of trypan blue would be of any value therapeutically in tuberculosis, a number of guinea-pigs were inoculated with a culture of human tubercle bacilli, and three days later, treatment was begun by subcutaneous injections of solutions of these salts.

Copper trypan blue.—On account of the necrotizing action of the copper, 0.5 and 1 c.c. of 0.1–0.25 per cent solutions of the dye were used.

1. A total of 42.5 mg. of dye or 5.44 mg. of copper was given in 28 injections. Pig died 77 days after inoculation. Local gland slightly enlarged but not caseous. Liver, spleen, and lungs filled with tubercles. Thick white exudate over pleura, pericardium, and peritoneum.

2. A total of 45 mg. of dye or 5.76 mg. of copper was given in 29 injections. Pig died 82 days after inoculation. Glands in axilla enlarged and caseous. Spleen, liver, and lungs filled with tubercles.

3. A total of 70.5 mg. of dye or 9 mg. of copper was given in 34 injections. Pig died 98 days after infection. Local gland slightly enlarged, not caseous. Liver, spleen, and lungs filled with tubercles.

4. A total of 46.5 mg. of dye or 5.95 mg. of copper was given in 29 injections. Pig died 103 days after infection. Local tubercle very small and not caseous. Spleen and liver large and necrotic. Lungs filled with tubercles.

5. Only 7 injections given and pig died of acute infection. No tuberculosis.

6. A total of 47.7 mg. of dye or 6.1 mg. of copper was given in 29 injections. Animal died 106 days after inoculation. No local tubercle. Spleen, liver, and lungs filled with tubercles.

Silver trypan blue.—The toxic dose of this salt having been determined by injections into mice, 0.5 c.c. of 0.1 per cent solution was decided upon as the maximum safe initial dose, this being later increased to 2 c.c. of a 0.5 per cent solution.

1. A total of 26 mg. of dye or 8.6 mg. of silver was given in 9 injections. Death occurred 23 days after inoculation. A local, slightly enlarged, but not caseous gland was the only indication of the development of the tuberculous infection.

2. A total of 103 mg. of dye or 34.3 mg. of silver was given in 18 injections. The pig died 44 days after infection, showing a local and general tuberculosis. The tubercles were all stained deep blue, while the normal tissue appeared unstained.

3. A total of 103.5 mg. of dye or 34.48 mg. of silver was given in 18 injections. Animal died 56 days after infection, with slight local and marked general tuberculosis. All tubercles were blue, normal tissue unstained.

4. A total of 138.5 mg. of the silver trypan blue or 46.1 mg. of silver was given in 21 injections. Death occurred 58 days after inoculation, autopsy showing slight local but very marked general tuberculosis. Tubercles were bright blue and all other tissues very red.

5. This pig received 22 injections of 140.5 mg. of dye, or 46.8 mg. of silver. Death occurred 58 days after infection, with slightly enlarged, non-caseous local glands, the spleen, liver, and lungs showing many definite and some caseous tubercles.

Iron trypan blue.—The initial dose, as in the silver trypan blue, was 0.5 c.c. of a 0.1 per cent solution, gradually increased to 2 c.c. of a 0.5 per cent solution.

1. A total of 149 mg. of dye or 11.77 mg. of iron was given in 23 injections. Death occurred 61 days after infection. Local glands were slightly enlarged, but non-caseous; spleen, liver, and lungs were filled with tubercles. All tubercles blue, the other tissues appearing unstained. No blue-stained bacilli seen.

2. This pig received 23 injections, totaling 149 mg. of dye or 11.77 mg. of iron. Death occurred 62 days after inoculation. Local glands were moderately enlarged and caseous; liver, spleen, and lungs markedly involved. All tubercles were deeply stained, even the caseous centers of tuberculous glands being blue.

3. A total of 189 mg. of the dye and 14.9 mg. of iron was given in 27 injections. The animal died 73 days after inoculation. A local gland was slightly enlarged and caseous, while liver, spleen, and lungs were filled with miliary tubercles and necrotic areas.

4. A total of 164 mg. of the dye or 12.95 mg. of iron was given in 28 injections. Death occurred 79 days after inoculation. Local glands slightly enlarged and hard, not caseous. Spleen large and necrotic. Liver but slightly involved. Lungs contained a few small tubercles. All tubercles and necrotic areas were blue.

5. A total of 209 mg. of the dye or 16.5 mg. of iron was given in 29 injections. The pig died 104 days after infection, showing a very small, hard, local tubercle. Spleen and liver were filled with tubercles and necrotic areas. The lungs were packed with tubercles, many containing cavities.

Mercury trypan blue.—In the therapeutic experiments, only the mercuric salt was used, the stock suspension being injected subcutaneously. The individual doses represented 0.2–0.8 mg. of Hg corresponding to 0.004–0.016 grain of $HgCl_2$ or 0.0035–0.014 grain of calomel.

1. A total of 10.56 mg. of dye or 3.33 mg. of Hg was given in 23 injections. Pig died 61 days after infection. Two slightly enlarged, but not caseous glands were found in groin. Spleen, liver, and lungs showed only a few small tubercles. The tuberculous process was not far enough advanced to be considered the cause of death. Neither tubercles nor normal tissues appeared stained with these dyes.

2. This pig received 26 injections, making 10.89 mg. of dye or 3.44 mg. of mercury, and died 71 days after infection. No local nodules. A few small tubercles in liver, spleen, and lungs. No dye seen anywhere.

3. This pig received 29 injections of 11.22 mg. of the dye and 3.54 mg. of mercury. Death occurred 87 days after infection. Autopsy showed slight enlargement of local glands; liver and spleen normal; lungs congested and presenting a few small tubercles. No dye seen.

4. Same amount and same number of injections as in No. 3. Death took place 117 days after inoculation. The local glands were slightly enlarged and the spleen and lungs well filled with tubercles.

5. In this animal, the dye injections were begun 20 instead of 3 days after infection. A total of 5.94 mg. of dye or 1.88 mg. of mercury was given in 23 injections. The pig died 142 days after inoculation. Autopsy showed slight local, but marked general tuberculosis.

It may be noted as curious that in all the animals treated with these salts of trypan blue, the local tuberculous process is either slight or absent, while the organic involvement is much more pronounced. It is difficult to say whether this marked variation from the ordinary course of development of tuberculosis in the guinea-pig is due to any action of the metals, possibly stimulating phagocytosis. As Table 3 shows, the animals treated with copper trypan

TABLE 3.

SHOWING DURATION OF LIFE IN THESE EXPERIMENTS.

Controls	Copper Trypan Blue	Silver Trypan Blue	Iron Trypan Blue	Mercuric Trypan Blue
Days	Days	Days	Days	Days
82	77	23 (not tuberculosis)	61	61
71	82	44	62	71
97	98	56	73	87
.....	103	58	79	107
.....	106	58	104	142
Av.....$76\frac{3}{4}$	$93\frac{1}{5}$	$47\frac{4}{5}$	$75\frac{4}{5}$	$97\frac{3}{5}$

blue and with mercuric trypan blue lived considerably longer than the controls and longer than the animals treated with the other salts. Of more importance seems the fact that the animals treated with the mercury salt, with the single exception of No. 5 in which the treatment was begun late, had a very slight tuberculous involvement, either local or general, and showed many symptoms suggesting chronic mercury poisoning.

The silver and iron salts of trypan blue penetrate the tubercle as well as or better than does the sodium salt; the normal tissue is nearly unstained and under the microscope shows but few of the cells filled with blue granules which are so characteristic of the

tissues of the trypan blue stained animals. The copper and mercury salts of the dye, on the other hand, have failed to penetrate the tubercle or to stain the normal tissues. Since the mercury salt is insoluble and was used in a fine suspension which settled out very slowly, it seemed not unlikely that this insolubility might be the reason for its slight diffusibility and for the fact that it has failed to stain any of the tissues. The copper salt, on the other hand, is readily soluble in water. At first it seemed possible that the necessarily minute doses might explain the lack of distribution. Later this explanation seemed insufficient and the thought occurred to me that the dye, on coming in contact with the body tissues, might flock out of solution, or at least become coarsely colloidal, in which state it might be less readily taken up by the cells and by the lymph. The marked necrotizing action of these salts was a factor strongly favoring the idea of retention at point of injection, and it was also found that egg albumin and pieces of fresh tissues added to a solution of the dye in a test tube caused a separation of the solution into two definite layers, a thick, heavy layer at the bottom and a clear layer at the top, which might be water-clear or somewhat stained. In order to discover what happened when the copper salt was injected subcutaneously, a lethal dose was injected into three pigs. One received 5 c.c., the second received 8 c.c., and the third had 10 c.c. of a 1 per cent solution. The guinea-pigs lived from 12 to 20 hrs. Within this time trypan blue would have disappeared from the point of injection and been distributed over the body. In these three pigs the organs were completely unstained and not even the skin beyond the natural spread of the fluid was stained, while a mass of soft gelatinous dark blue substance filled the entire groin and saturated the subcutaneous tissues in this region, but had not even penetrated to the underlying muscle. The failure of the copper dye to distribute itself seems, therefore, to be due not so much to the small doses as to the nature of the dye compound, which is such that it falls out of solution when it comes in contact with the protein of the tissues. A similar fact was noted by Corper[1] who observed that soluble copper salts became colloidal on coming in contact with living tissues.

[1] Wells, DeWitt, and Corper. Unpublished manuscript, to appear in the *Ztschr. f. Chem.*

Some attempts have been made to determine whether these heavy metal salts of trypan blue were carried into the internal organs as such or even whether the metals were carried in at all. First the tissues from a pig treated with copper trypan blue and those from a pig treated with silver trypan blue were extracted by Dr. Corper in an effort to analyze the extracted dye for the metals. No copper, or not sufficient copper for determination, was found in the organs of the pig treated with copper; and in the silver trypan blue tissues the dye was found so firmly fixed in the cells and tissues that it could not be removed by any of the methods used. I have therefore tried certain biochemical methods for determining the presence of these metals. The fresh tissues of a guinea-pig treated with silver trypan blue were dropped into the pyrogallic acid-formalin mixture used in the Cajal and other methods for the reduction of silver in the tissues. Other tissues were fixed in Zenker's solution, which removes or fades the dye, and were then examined for silver granules in the tissues. By neither of these methods was I able to determine with any degree of certainty the presence of silver, either in the tuberculous or in the normal tissue. Sections of alcohol-fixed tissues from a pig treated with copper trypan blue were placed, some in potassium ferrocyanid, which should stain the copper-containing parts red, some in solution of ammonium hydrosulfide, in which the copper-containing parts should become yellowish brown, and some in dilute water solution of hematoxylin, which should stain the copper-containing parts deep blue. By none of these methods was I able to find a trace of copper. While the iron trypan blue was usually quickly absorbed and distributed over the body, in some of the animals long treated with this salt, especially if the solutions were not always fresh, a reddish deposit suggesting colloidal iron was found around the point of injection. Sections of tissues from guinea-pigs treated with iron trypan blue were exposed to the ammonium hydrosulfide and potassium ferrocyanid as suggested by Macallum, in which the iron-containing parts should appear green to black after the sulfide, but become blue after the ferrocyanid. Since all of these methods have been negative, it seems probable that these salts of trypan blue are easily broken

down and that the metals are not carried by the dye to the internal organs. This fact may explain the failure to obtain any favorable therapeutic results from the use of these heavy metal salts of trypan blue.[1]

SUMMARY AND CONCLUSIONS.

Trypan blue and trypan red readily penetrate the tubercle in all stages of its development, thus showing that it is possible to penetrate the avascular tubercle by chemicals introduced either subcutaneously, intravenously, or intraperitoneally.

Trypan blue and trypan red do not penetrate the tubercle bacillus well, and do not kill it *in vitro* even after 24 hours' exposure to a 1 per cent solution.

In therapeutic doses, frequently repeated for long periods, trypan blue and trypan red seem to have no favorable or curative influence in experimental tuberculosis in guinea-pigs. In a single large, nearly lethal dose at the beginning of the infection they also have no favorable influence.

Silver trypan blue and iron trypan blue also penetrate the tubercle, but have no bactericidal and no therapeutic influence. It is doubtful whether the metals are carried in with the dye.

Copper trypan blue is soluble, but does not penetrate either the normal or the tuberculous tissues, and is probably changed to an insoluble form or a suspension colloid and retained at the point of injection.

Mercury trypan blue is insoluble, is strongly bactericidal in its action on the tubercle bacillus, but is too toxic for therapeutic use, since the pigs died apparently from chronic mercury poisoning rather than from the tuberculous infection, the tuberculous process being generally very slight. The findings with this salt, however, are suggestive, and further experiments with mercury salts will be made.

[1] It gives me great pleasure to express my indebtedness to Dr. Walter Fraenkel for his assistance in making these metal salts and to Miss Sherman for the faithfulness and interest with which she has aided me in much of the tedious routine of this experiment.

COMPARISON OF THE PLATING AND MICROSCOPIC METHODS IN THE BACTERIOLOGICAL EXAMINATION OF MILK.*

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Bacterial counts, as a means of milk supply control, have been made but a few years, but in this short time their use has been almost universally adopted.

The plating method, the original method, is today the standard. This wide use has shown it to be open to one very serious objection, in that the incubation period required for the growth of colonies is from 24 to 48 hrs. Within this period, under ordinary conditions, the milk should be marketed and consumed. Hence there has been a demand for a simpler and quicker method.

In 1905, Dr. Frances H. Slack[1] read before the Society of American Bacteriologists a paper giving an account of a method which he had developed for making bacterial counts by means of estimating the number of bacteria in a smear. He gives the following account of his discovery of the method:

"The writer, in carrying out routine examinations of milk sediments for pus and streptococci, noticed that the number of bacteria found in the microscopic field apparently bore a definite relation to the number of colonies developing in the plate from the same sample. It was thought that this might be of practical value if, after prolonged comparison, it turned out to be constantly true, since there would then be no necessity to plate, incubate, and count samples which the microscope alone indicated were better than the legal requirements. . . . . It was decided to test the method thoroughly, comparing the microscopic estimate with the actual count obtained from the plate. This comparison was carried out very carefully with over 2,200 samples, each sample being subjected to the double test—i.e., centrifugalizing and plating, the microscopic estimate being made before the plate was counted, usually within a few hours after the samples were received—and an error of less than 1 per cent was made in passing as below 500,000 bacteria to the cubic centimeter milks which in the plates showed above this limit. Over a third of the total error occurred in the first 420 samples before the method was fully developed."

Slack used a factor of 10,000 in his estimate of the number of bacteria in a smear. We quote the following, showing how he arrived at this particular number:

"We may say as a rough estimate that each coccus, bacillus, diplococcus, or chain in the 1/12 oil immersion field represents one colony in the 1/10,000 plate from the same sample. The writer wishes to emphasize the fact that this is a coincidence

* Received for publication July 3, 1913.

[1] *Technology Quarterly*, 1906, 19, p. 1.

stumbled upon in the course of routine work rather than a carefully worked out mathematical result. It is, of course, impossible to obtain a perfectly even smear, and the sample must be sized up by examining a number of fields. Let the observer find such a representative field, then imagine that instead of looking at a microscopic field through a 1/12 oil immersion lens he is looking at his 1/10,000 dilution plate, and that each coccus, bacillus, diplococcus, or chain within his vision represents a colony on such a plate; and if he will make the plates also, he will find how closely in the main they agree with his microscopic estimate. That is, in most cases the count of a representative field multiplied by 10,000 gives approximately the number of bacteria per cubic centimeter."

Such is the demand for a better method than the plating method for bacterial counts that the microscopic method is beginning to receive official recognition. However, the method has been modified so as to change the factor from 10,000 to 20,000. Certain firms of instrument makers are manufacturing special centrifuges for this particular work. One of these is in use in the laboratory of the state food chemist at the University of Wyoming.

Since Slack reports his work on the basis of 500,000 bacteria to the cubic centimeter, and since his original factor has been changed, and since, further, he has never published his exact ratio between the counts of the two methods, the writer deemed it worth while to undertake a series of experiments for the purpose of verifying Slack's work and obtaining some data on the reliability of the microscopic count for proving that milk is up to a standard of 100,000.

The milk samples used in the following tests were taken from cows at the University Stock Farm. Samples were secured in the morning and the microscopic examinations and plates were usually made about 2:00 P.M.

After counting the number of bacteria present in 5 or 6 of the most representative fields and then taking the average of the counts of all these representative fields, this result was multiplied by the 20,000 factor. After various trials it was found that methylene blue was the most satisfactory stain, and this was used in all cases except where otherwise stated.

The tabulations giving the 430 bacterial counts of the two methods, in which the 20,000 factor was used in the microscopic estimation and a 1/1,000 dilution and 48-hour incubation period in the plating method, have been omitted, since all computations have been based on the averages of the counts in groups of 50. Table 1 shows the averages of the bacterial counts by groups of 50, together with the ratio of the microscopic counts to the plate counts.

Even the most superficial examination of Tables 1 and 2 shows that there is a close relation between the counts by the plate and by the microscopic method. In Table 3, where the averages of the groups are compared, this close relation is very apparent. We have worked out the ratio between the averages, dividing the microscopic count by the plate count. These averages throw some light on the problem of whether or not 20,000 is the best factor to use in making the microscopic estimate. Taking the general averages of all the counts, we find that in round numbers the figures

for the microscopic and plate methods are identical. The resulting ratio of 1 shows that 20,000 is the best possible number to use for the factor. However, a study of the group averages shows that the ratios greater than 1 occur in the first three groups, while the remainder are all less than 1, the lowest being 0.76. Allowing

TABLE 1.

Group	No. of Samples	Microscopic	Plate	Ratio of Microscopic to Plate	Date
A	50	163,000	116,000	1.40	Sept.
B	"	138,000	134,000	1.03	Sept.–Oct.
C	"	86,000	78,000	1.10	Oct.
D	"	178,000	185,000	0.96	"
E	"	84,000	91,000	0.92	Nov.
F	"	83,000	97,000	0.85	"
G	"	70,000	92,000	0.76	Nov.–Jan.
H	"	67,000	83,000	0.80	Feb.
I	30	82,000	88,000	0.93	"
Gen. average	.....	107,000	107,000	1.00	.....
Groups A and B excluded.					
Gen. average	.....	97,000	102,000	0.95	.....

TABLE 2.

SHOWING ALL THE SAMPLES IN WHICH THE RATIO OF THE MICROSCOPIC TO THE PLATE COUNT WAS LESS THAN 0.5 OR GREATER THAN 2.

Date	Cow No.	Microscopic	Plate	Ratio Mic./Plate	Group
Sept	162	50,000	100,000	0.50	A
Oct	100	40,000	100,000	0.40	B
"	162	100,000	50,000	2.00	C
Nov	134	60,000	200,000	0.30	E
"	107	100,000	25,000	4.00	"
"	134	75,000	35,000	2.10	"
"	134	120,000	60,000	2.00	"
"	128	55,000	125,000	0.44	"
"	100	125,000	280,000	0.41	"
"	128	115,000	300,000	0.38	"
"	117	100,000	200,000	0.50	F
"	130	40,000	80,000	0.50	G
Jan	100	30,000	125,000	0.24	"
"	107	60,000	150,000	0.40	"
"	109	50,000	25,000	2.00	"
"	117	75,000	200,000	0.37	"
"	128	40,000	80,000	0.50	"
"	135	100,000	200,000	0.50	"
"	136	175,000	300,000	0.55	"

for the possibility of greater error at the beginning of the work, due to technic, it has been considered desirable to get the ratio of the average counts after throwing out the first two groups. This ratio is 0.95, which indicates that the factor 20,000 is a trifle too low. If it is assumed that 0.95 is the true ratio, then 21,050

would be the correct factor. In view of the small number of data on which the ratio 0.95 is based, together with the small error which that shows in the conventional factor, it is doubtful if anything would be gained by changing it. Altho, as shown in the preceding paragraph, the accuracy of the microscopic method could be but little improved by changing the factor, one must not lose sight of the fact that the milk inspector is concerned with individual samples and not with general averages. To show the variation of the individual counts of the microscopic method as compared with the plate method, Table 2 has been compiled, giving all the samples in which the ratio of the microscopic to the plate count was less than 0.5 or greater than 2.

TABLE 3.

SHOWING COUNTS WHICH DIFFER FROM THE PLATE COUNT BY 10,000 OR LESS.

	GROUP									TOTALS
	A	B	C	D	E	F	G	H	I*	
Positives	12	28	36	26	23	18	28	25	25	221
Percentage	24	56	72	52	46	36	56	50	83	51

* Group I, 30 samples.

The total number of samples in Table 2 is 19, which is 4.4 per cent of the total number tested. The highest ratio is 21 and the lowest 0.24. While these extreme ratios are not so large as to cast any doubt on the correlation of the microscopic to the plate counts, yet if it is considered that the ratios included in Table 2 indicate negative results by the microscopic method then there are 4.4 per cent of negatives, and a method which shows 4.4 per cent negatives should, to say the least, be used with extreme caution. Nor is this percentage of assumed failures lessened if the first two groups are thrown out, for they have only 2 per cent of samples in Table 2.

It is doubtful, however, in practical milk control, whether all microscopic tests which show a ratio between 0.5 and 2 could be considered as positive. Perhaps the widest variation from the plate count which could be included in the list of positives would be a count not differing more than 10,000 from the plate count. The number of positives found in each group is given in Table 3.

A study of Table 3 shows that if this high standard for positive results is adopted the microscopic method must fail, for it appears that according to this standard there are only 51 per cent positives. Group I, which has the highest percentage of positives, 83 per cent, contains only 30 samples. The highest percentage with full groups of 50 samples is 72 per cent, in Group C. Group A contains the lowest percentage of positives, 24 per cent. If the first two groups be again excluded to lessen the possibility of error, the percentage of positives in the remaining groups gains only slightly over the percentage when all the groups are considered. Total number of positives when groups A and B are excluded is 55 per cent. Since a method which shows only 55 per cent of positive results must be considered a failure, either the standard has been set too high or the microscopic method is not efficient.

The problem of the value of the microscopic count for milk control may be approached from another direction. As milk control is ordinarily carried out some arbitrary standard is fixed and all milk which shows a bacterial count no greater than this standard is regarded as "passed." With this fact in mind a study of the data has been made to determine how often milk samples would have been wrongly "passed," or unjustly "not passed" by the microscopic counts. In making this study two standards were used: first 50,000, and second 100,000. Altho in the control of market milk, standards of 500,000 or higher are used, the samples studied in this paper ran so uniformly low in count that nothing would be gained for the purpose of this discussion by taking a standard higher than 100,000.

Table 4 shows how the microscopic method would have worked in connection with the samples studied, if a count of 50,000 or less had been required for regarding the milk as "passed." Table 5 shows the microscopic method with 100,000 as a standard for passing.

TABLE 4.

Group	Number of Samples	Passed by Both Methods Pp	Not Passed by Both Methods Nn	Passed by Plate but Not by Microscopic Method Pn	Not Passed by Plate, but Passed by Microscopic Method Np
A	50	21	14	14	1
B	"	15	30	2	3
C	"	10	32	8	0
D	"	4	43	1	2
E	"	1	38	11	0
F	"	0	42	1	7
G	"	5	36	0	9
H	"	1	38	0	11
I	30	0	29	0	1
Totals	430	57	302	37	34
Totals exclusive of A and B	330	21	258	21	30

The total number of positives (Pp+Nn) was 359; excluding A and B, 279. The total number of negatives (Pn+Np), 71, excluding A and B, 51. Percentage of negatives was 16.5 per cent; excluding A and B, 15.4 per cent. The total number passed by microscopic method (Pp +Np) was 91; excluding A and B, 51. The total number passed incorrectly by microscopic method (Np) was 34; excluding A and B, 30. Np was 37 per cent of (Pp+Np); excluding A and B, 58 per cent. The total number not passed by microscopic method (Nn+Pn) was 339; excluding A and B, 279. The total number not passed incorrectly by microscopic method (Pn) was 37; excluding A and B, 21. Pn was 11 per cent of Nn+Pn; excluding A and B, 7.5 per cent.

TABLE 5.

SHOWING THE WORKING OF THE MICROSCOPIC METHOD AS COMPARED TO THE PLATE METHOD IN PASSING OR NOT PASSING MILK, AS NOT BEING OF HIGHER THAN 100,000 BACTERIAL COUNT.

Group	Number of Samples	Passed by Both Methods Pp	Not Passed by Both Methods Nn	Passed by Plate, but Not by Microscopic Method Pn	Not Passed by Plate, but Passed by Microscopic Method Np
A	50	45	5	0	0
B	"	37	12	0	1
C	"	38	10	1	1
D	"	22	22	3	3
E	"	33	12	2	3
F	"	31	16	0	3
G	"	37	6	0	7
H	"	42	0	0	8
I	30	26	3	0	1
Totals	430	311	86	6	27
Totals exclusive of A and B	220	229	69	6	26

The total number of positives (Pp+Nn) was 397; excluding A and B, 298. The total number of negatives (Pn+Np), 33; excluding A and B, 32. The percentage of negatives was 7.6 per cent; excluding A and B, 7.4 per cent. The total number passed by microscopic method (Pp+Np) was 338; excluding A and B, 255. The total number incorrectly passed by microscopic method (Np) was 27; excluding A and B, 26. Np was 8 per cent (Pp−Np); excluding A and B, 10.2 per cent. The total number not passed by microscopic method (Nn−Pn) was 92; excluding A and B, 75. The total number incorrectly not passed by microscopic method (Pn) was 6; excluding A and B, 6. Pn was 6.5 per cent of (Nn−Pn); excluding A and B, 8 per cent.

Table 4 shows that with the "passing" standard of 50,000, for the whole number of 430 samples the total number of negatives was 71, or 16.5 per cent, and that if Groups A and B were excluded because of error in technic the percentage of negatives becomes 15.4 per cent. Table 5 shows that with a "passing" standard of 100,000 the percentage of failures in the whole number of counts was 7.6 per cent, and with Groups A and B excluded, 7.4 per cent.

It is of great importance to the man engaged in practical milk control, who has to face the possibility of carrying his cases to court, to know of a given method how many samples would be unjustly "not passed" out of the total number "not passed." Table 4 shows that, with the samples in question and the passing standard 50,000, the microscopic method would have unjustly "not passed" 37 out of a total of 339 "not passed," making a percentage of negatives 11 per cent; if the Groups A and B are excluded the percentage becomes 7.5 per cent. Table 5 shows that if the "passing" standard is 100,000 the percentage incorrectly "not passed" would have been 6.5 per cent, and, if the Groups A and B were excluded, the microscopic method would have shown 8 per cent of negatives.

All this shows that at least where a high "passing" standard is used, injustice would be done by condemning milk on the microscopic count on a single slide. Moreover, it is certain that a method with such a high percentage of negatives would have no standing in the courts. It seems that this method could be used for the control of market milk only when a check was provided by making a number of slides from each sample or by checking all the "not passed" counts by the plate method. The total number incorrectly "passed" as compared to the total number "passed" is not of so much importance as appears at first sight. This is especially true where a comparatively high "passing" standard is used. While from Table 4 with the "passing" standard at 50,000 the number incorrectly "passed" is 37 per cent of the total number "passed" yet it is shown in Table 5 that in lowering the standard to 100,000 the number incorrectly "passed" was reduced to 8 per cent of the total number "passed." Now it is doubtful that a count of 200,000 would be considered dangerous if the "passing" standard be held at 100,000 or even 50,000. When it is remembered in this connection that the highest ratio of microscopic to plate count was found to be but 2.1 per cent and that ratio occurred only once in 430 times, it will be seen that the chance of "passing" a milk with a count of 200,000, when the standard is 100,000, is extremely small even when it is regarded as "passing" on the microscopic count of a single slide; and with the standard at 50,000

the chance of "passing" a milk with a count of 100,000 is just as small. So far as is shown by the data of this paper it would be an impossibility to "pass" a milk with a plate count of even 300,000 by using the microscopic method with a "passing" standard of 50,000.

From this the value of the microscopic method in the milk trade is apparent. A milk that is found by experiment to have a microscopic count of say 100,000 or less could be depended upon to be fairly clean milk, while that which did not pass this standard would no doubt be sold as ordinary market milk so that the 8 or 10 per cent unjustly "not passed" would not be a total loss to the producer.

It must not be forgotten that these discussions of the microscopic count are based on the counting of single slides, but if the milk were sampled in duplicate or triplicate it is evident that the counts which checked would be likely to be found more accurate as compared with the plate counts than those studied in this paper.

SUMMARY.

The results of this work point to the following conclusions:

1. That there is a marked correlation between the plate and microscopic counts.

2. That little, if any, improvement can be made on the factor 20,000 which is used to reduce the microscopic count to terms of the plate count.

3. That the microscopic count from a single slide can be depended upon as being within the limits of one-third as great as three times as great as the plate count.

4. That the microscopic count from a single slide cannot be depended upon as being within 10,000 of the plate count.

5. That the microscopic count from a single slide is not sufficiently reliable to warrant the condemnation of market milk, especially when the standard for passing is a low count.

6. That any milk which is "passed" on the microscopic count from a single slide, where the "passing" standard is a low count, is not likely to have a dangerously high count by the plate method.

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